Haemolytic Activity of *Escherichia coli*

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To my Father,
for many things.

Science is a first rate piece of furniture
For a man's upper chamber
If he has common sense on the ground floor.

Oliver Wendell Holmes.

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OBJECT OF THE RESEARCH

In the past decade an intensive search has been made to establish the role of Escherichia coli enterotoxins as causative agents of diarrhoeal disease in man and domestic animals. Indeed, enough evidence now exists to conclude that enterotoxin is responsible for the symptoms of this disease.

E. coli strains also produce at least one haemolysin. However, the cultural conditions necessary for production of haemolysin are poorly understood. Also, highly purified haemolysin has not previously been reported and there is little information about the role of haemolysin in pathogenicity. In view of these deficiencies the object of this research was:

1. To survey the incidence of haemolytic E. coli isolated from inpatients and outpatients at a Glasgow hospital.
2. To produce E. coli haemolysin in large quantities.
3. To purify and characterise E. coli haemolysin.
4. To study the relationship between cell-associated and extracellular haemolysin.
5. To investigate the kinetics of haemolysin activity and, if possible, find a single substrate for the haemolysin.
6. To study the biological properties of E. coli haemolysin.

Studies of the role in pathogenicity of cytolytic bacterial toxins are not meaningful using crude preparations. Therefore, purification of E. coli haemolysin was the major aim of this research.

Contents

Results

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF PLATES

LIST OF ABBREVIATIONS

BHK	=	baby hamster kidney.
BSA	=	bovine serum albumin, fraction V.
CDM	=	chemically defined medium (Appendix I).
cm^2	=	square centimetre.
^oC	=	degrees centigrade.
D_{20}	=	diffusion coefficient.
DEAE	=	diethylaminoethyl.
DNA	=	deoxyribonucleic acid.
E	=	extinction.
EDP	=	oedema disease principle.
EDTA	=	ethylenediaminetetraacetic acid.
g	=	gram or acceleration due to gravity.
hr	=	hour
HSA	=	human serum albumin.
HU	=	50% haemolytic unit as determined spectrophotometrically.
HU_{50}	=	50% haemolytic unit as determined visually.
in^2	=	square inch.
kg	=	kilogram.
Kv	=	kilovolts.
lb	=	pound.
LPS	=	lipopolysaccharide.
LT	=	heat-labile enterotoxin.
M	=	molar.
μ	=	microns (see also nm).
mAmp	=	milliampere.
MEB	=	meat extract broth medium (Appendix I).
μg	=	microgram.

mg	=	milligram.
min	=	minute.
ml	=	millilitre.
mm	=	millimetre.
mM	=	millimolar.
M.W.	=	molecular weight.
N	=	normal (solution) or Avogadro's number.
NBG	=	nutrient broth-glucose medium.
NCTC	=	National Collection of Type Cultures.
nm	=	nanometres.
pH	=	negative logarithm (base 10) of hydrogen ion concentration.
pI	=	iso-electric point.
rev	=	revolutions.
RNA	=	ribonucleic acid.
SDS	=	sodium dodecyl sulphate.
sec	=	second.
SRBC	=	sheep red blood cells.
ST	=	heat-stable enterotoxin.
TCA	=	trichloroacetic acid.
TLC	=	thin layer chromatography.
Tris	=	Tris (hydroxymethyl) aminomethane.
U.V.	=	ultraviolet.
V	=	volts or volume (of a sphere).
$\bar{V}$	=	partial specific volume.
V-C	=	veronal-calcium chloride buffer (Appendix II).
V_o	=	void volume.
v/v	=	volume for volume.
w/v	=	weight for volume.

INTRODUCTION

INTRODUCTION

A. The Pathogenicity of Escherichia coli.

1. General remarks.

The organism now known as Escherichia coli was first described by Escherich in 1885. A similar organism was isolated from a diarrhoeal disease of calves by Jensen in 1893 who noted that this disease had been described at least 100 years previously. From the early 1900's E. coli became recognised as a characteristic member of the flora of the intestinal tract of vertebrates. The biochemical characteristics of the type species of the genus Escherichia have been detailed by Breed, Murray and Hitchens (1948) and the work of Kauffmann and others (see Kauffman, 1966) in the last 30 years has established that members of the species, E. coli, can be further classified by serological methods (see section A:2).

E. coli has been identified as the causative organism in certain cases of septicaemia, meningitis, peritonitis and pyelonephritis, and is a common cause of infections of the genito-urinary tract. The organism is associated also with enteric infection in man and related diseases in domestic animals.

Various terms, 'colibacillosis', enteric infection, gastroenteritis, neonatal or infantile diarrhoea, diarrhoeal disease, 'travellers diarrhoea' and 'white scours', have been used to describe a localized infection of the small intestine which results in an outpouring of fluid and concomitant dehydration of the body tissues. In general, the clinical term reflects the particular animal or age group which is affected.

The purpose of this introduction is to present a critical assessment of the role of E. coli and its products in enteric disease.

2. The serology of *E. coli*.

A brief resume of the serological classification of *E. coli* is necessary since much of the literature relating to the organism as an enteric pathogen concerns the isolation of specific serotypes (Kauffmann, 1966).

Historically, the identification of individual strains of *E. coli* was hampered by the lack of suitable serological techniques. Early attempts (Durham, 1896-97; Mackie, 1913; Dudgeon, Wordley and Bawtree, 1921; 1922) were of limited value and only served to show that *E. coli* was antigenically complex.

Kauffmann (1943) introduced a reliable method of serological typing based on agglutination analysis of somatic, O antigens, capsular or envelope, K antigens and flagellar, H antigens. In 1947, Kauffmann proposed a scheme for the classification of *E. coli* serotypes based on his own earlier studies (Kauffmann, 1944a; 1944b; 1944c) and on those of Knipschildt (1945; 1946) and Vahlne (1945). This scheme was extended (Ewing *et al.*, 1956; Ewing and Davis, 1961) and the serological classification of *E. coli* has been presented in detail by Kauffmann (1966).

The O antigens are composed mainly of polysaccharide and lipid and form an integral part of the lipopolysaccharide (LPS) or endotoxin complex of gram-negative bacteria (for reviews of *E. coli* LPS and endotoxin, see Heath *et al.*, 1966; Osborn, 1969; Weinbaum, Kadis and Ajl, 1971). Flagellar, H antigens may or may not be present and are of minor importance. The presence of K antigens can cause inagglutinability of O antigens by homologous antisera, a property which might account for difficulties in serotyping *E. coli* experienced by early workers. Indeed, Smith and Bryant (1927) found that, under different cultural conditions, colonies of *E. coli* appeared lacking the antigenic characters which prevented O agglutination. Later work confirmed

that O-inagglutinability was due to the presence of K antigens and 3 forms were recognised by susceptibility to heat (Table 1). It is current practise to describe the antigenic composition of a particular strain in the following way; 055:K59(B5):H7. Strains of E. coli are grouped according to their O antigens; thus 055 is a particular serogroup. A complete analysis of O, K and H antigens identifies a serotype.

Recently, Ørskov et al., (1967) conducted an extensive immunochemical survey of the sugar composition (chemotype) of E. coli LPS prepared from most of the known serogroups; the 100 serogroups examined corresponded to 28 chemotypes. Analysis of the sugar composition of hydrolysates of LPS may in future provide an alternative approach to the classification of E. coli.

3. The causes of enteric disease.

E. coli as an enteric pathogen: Within the Enterobacteriaceae, enteropathogenic E. coli, along with Salmonella and Shigella species are considered as the major bacterial pathogens associated with enteric disease, especially in infants. However reports in the past 20 years from Europe and North America show that these pathogens have been isolated from a maximum of 38% (mean, 18%) of hospitalised patients with this condition (Wegman, 1955; Walker et al., 1960; Yow et al., 1963; 1966; 1970; Stroebel and Cramblett, 1968; Moffet, Schulenberger and Burkholder, 1968; Cramblett, Azimi and Haynes, 1971). Table 2 shows that, in at least 62% of cases of infantile diarrhoea, no bacterial pathogen was isolated. Indeed, in most reported cases no recognised pathogen was isolated. It can be seen that the main viral pathogens were ECHO, Adeno, Coxsackie and Polio viruses. Also, combined viral and bacterial infections accounted for a significant number of cases. The protozoan parasites, Entameba histolytica and Giardia lamblia (Browning and Mackie, 1949; Gordon, 1971) were isolated only rarely.

Table 1. Characteristics of K antigens of *E. coli*[1].

K Antigen Type		Characteristics
L	1.	Envelope origin.
	2.	Agglutinogen destroyed by heat at 100°C for 1 hr.
	3.	Antibody-binding property thermolabile.
	4.	Rarely found in O groups 8 and 9.
B	1.	Envelope origin.
	2.	Agglutinogen destroyed by heat at 100°C for 1 hr.
	3.	Antibody-binding property thermostable.
	4.	Often found in enteropathogenic serogroups.
C	1.	Capsular origin.
	2.	Agglutinogen thermostable at 121°C for 2.5 hr.
	3.	Antibody-binding property thermostable.
	4.	Usually found in O groups 8 and 9.

1. After Kauffmann (1966).

Table 2 : Aetiologic agents of human infantile diarrhoea

Agent	Range (%) of Cited Isolations	Mean (%) Isolation	References
Bacteria			
Enteropathogenic			
E. coli	(1 - 23)	10.5	Wegman, 1955; Walker et al., 1960;
Salmonella	(1.8 - 11)	4	Yow et al., 1963; 1966; 1970;
Shigella	(0 - 10.7)	3	Stroebel and Cramblett, 1968;
	Total	17.5	Moffet et al., 1968; Cramblett et al., 1971.
Viruses			
ECHO	(2.5 - 13.1)	8.5	Walker et al., 1960; McLean, McNaughton
Adeno	(4 - 17)	8.2	and Wyllie, 1961; Lepine et al., 1963;
Coxsackie	(0.5 - 6.6)	2.6	Yow et al., 1963; 1966; Moffet et al.,
Polio	(0 - 2)	1.5	1968; Cramblett et al., 1971.
Others	?	?	
	Total	20.8	
Bacterial/Viral	(1 - 22)	11	Sommerville, 1958; Ramoz-Alvarez and Sabin, 1958; Gardner, McGregor and Dick, 1960; Young et al., 1962; Lepine et al., 1963; Ramoz-Alvarez and Olarte, 1964; Guardiola-Rotger et al., 1964; Cramblett and Siewers, 1965; Yow et al., 1970; Cramblett et al., 1971.
Protozoan	?	?	Browning and Mackie, 1949; Gordon, 1971.
No pathogen isolated	(37 - 96)	50	Walker et al., 1960; Lepine et al., 1963; Yow et al., 1963; 1966; Moffet et al., 1968; Cramblett et al., 1971.

E. coli strains are found commonly in the bacterial flora of the intestines of normal individuals. Although more than 140 serogroups of E. coli are known, until 1970 only 17 of these had been isolated from cases of diarrhoeal disease in man (Table 3). Similarly a small percentage of serogroups are responsible for the equivalent disease in domestic animals (Glantz, 1971).

So-called enteropathogenic serotypes have been isolated with varying frequency from healthy individuals (Ørskov, 1956; Ewing, 1962; Bettleheim and Taylor, 1971). Taylor (1966) has suggested that the extent to which pathogenic serotypes occur in normal subjects depends on whether the disease is endemic or epidemic in the population.

What factor(s) determine(s) the enteropathogenicity of E. coli? Certainly host factors, such as age and immunity to infection together with the level of socio-economic development in the community are significant factors (Aschaffenburg et al., 1951; Ross and Dawes, 1954; Morse, Furness and Meter, 1956; Svirsky Gross, 1958; Meter, 1959; Payne and Marsh, 1962; Gay, 1965; Gay et al., 1965; Kohler and Bohl, 1966; Taylor, 1966; Kohler, 1967; Mata, Urrutia and Garcia, 1967; Glantz and Jacks, 1969; Rowe, Taylor and Bettleheim, 1970; Gordon, 1971; Mata and Urrutia, 1971; South, 1971). However, the following review is restricted to a discussion of the products of E. coli which may play a role in pathogenicity.

Initiation of infection: In the important initial phase of enteric infection, i.e. proliferation of the organism, it is necessary for E. coli to bind to the small intestinal epithelium (Smith and Halls, 1968b; Arbuckle, 1970). Recent evidence indicates that the filamentous protein antigen K88 (Ørskov et al., 1961; 1964; Stirm, Ørskov and Ørskov, 1966; Stirm et al., 1967a; 1967b), found

Table 3 : Serotypes of *E. coli* isolated from human gastroenteritis to 1970

O Serogroup	K Antigen	H Antigen	Non Serological Designation[1]	Author
111	B4	H2;H12	D433	Bray, 1945; Taylor, Powell and Wright, 1949.
	B4	H-	Aberdeen α	Taylor, PHLS[2], 1950.
55	B5	H6;H7	Aberdeen β	Giles, Sangster and Smith, 1949; Holzel, Martyn and Apter, 1949; Smith, 1949; 1953; Taylor, 1951.
26	B6	H11;H-	E893	Ørskov, 1951; Charter and Taylor, 1952.
119	B14	H18;H27		Smith, 1953; Thomson, 1956.
44	K74(L)	H18		Ewing, 1962.
86	B7	H-	E990	Charter and Taylor, 1952.
112	B13;B11	H18;H-		Ewing, 1962.
114	?	H32		Report, 1969; Jacobs *et al.*, 1970.
124	B17	H30	R411	Hobbs, Thomas and Taylor, 1949; Ewing, 1953.
125	B15	H19	Canioni	Charter and Taylor, 1952.
126	B16	H2	E611	Charter and Taylor, 1952.
127	B8	H-		Ewing, 1962.
128	B12	H2		Ewing, 1962
	B12	H-	E6912	Taylor, PHLS, 1956.
142	K86	H6		Ørskov *et al.*, 1960; Olarte and Ramos-Alvarez, 1965; Love *et al.*, 1972.
148	?	H28		Taylor, PHLS, 1969.
18	B21	?		DTI[3], 1968.
20	B7	H-		DTI, 1968.

1. Some isolates given numerical or name coding before serotype was known.
2. PHLS: Public Health Laboratory Service, Colindale, London.
3. DTI: Difco Technical Information Sheet No. 0154 (Difco, Detroit, U.S.A.).

in porcine strains, enables these strains to adhere to and proliferate in the small intestines of piglets (Smith and Linggood, 1971b). Removal of this antigen by agitation in a blender (Stirm et al., 1967b) or by "curing" the plasmid which governs its production (Smith and Linggood, 1971b) makes the organism avirulent.

No corresponding agent of attachment has been isolated from human enteropathogenic serotypes. The mechanism which allows human strains to proliferate in the small intestine is not understood.

B. Toxins of E. coli

1. Endotoxin.

General biochemistry and toxicity: Endotoxin is a thermostable component of the cell envelope of gram-negative bacteria and it is generally accepted that the complex is composed of lipid, polysaccharide and protein moieties (Boivin, Mesrobeanu and Mesrobeanu, 1933; Morgan and Partridge, 1940; 1941; 1942; Goebel, Binkley and Perlman, 1945; Binkley, Goebel and Perlman, 1945; Ikawa et al., 1952; Rathgeb and Sylven, 1954; Westphal and Lüderitz, 1954; Webster et al., 1955; Ribi et al., 1960; Nowotny et al., 1963; Bishop and Work, 1965; Adams, 1967; Marsh and Crutchley, 1967; Lüderitz et al., 1971; Wober and Alaupovic, 1971). There is some confusion as to which portion of the complex is responsible for the observed biological effects. Removal of a high proportion of the protein component does not significantly alter the serological or toxic properties of endotoxin (Hartwell et al., 1943; Ikawa et al., 1952; 1954; Westphal, Lüderitz and Bister, 1952; Webster et al., 1955; Ribi et al., 1960) although reduced immunogenicity has been noted following deproteinisation (Westphal and Lüderitz, 1954).

Several laboratories (Westphal and Lüderitz, 1954; Kasai, 1966; Kim and Watson, 1967; Kasai and Nowotny, 1967) have shown for Salmonella species that only KDO (2-keto-3-deoxyoctonate) and Lipid A are necessary for endotoxic activity. Recently, Galanos et al., (1971) demonstrated that free lipid A, made water-soluble by complexing with bovine serum albumin, was by itself a potent endotoxin. They suggested that KDO may confer hydrophilic properties on the otherwise hydrophobic lipid A molecule. A similar function has been indicated for the protein component of native endotoxin (Wober and Alaupovic, 1971).

Despite many reported physiological and toxic effects of E. coli endotoxin (Weinbaum et al., 1971) its role in pathogenicity is unclear. Wide variations exist in the virulence for mice of E. coli strains isolated from different clinical conditions (Rowley, 1954; Erlandson, Nemer and Pearson, 1964; Medearis, Camitta and Heath, 1968) including isolates from enteric infections; endotoxin preparations from these strains also differed in their lethal activity for mice. Therefore, no definite correlation has been found between endotoxic activity and the clinical source of the strain.

E. coli endotoxin in enteric infection: A few isolated reports give conflicting views regarding the implication of endotoxin in enteric infection. Berczi (1968) and Berczi et al., (1968) noted that orally administered E. coli endotoxin prepared by phenol-water extraction (Westphal and Jann, 1965) was not absorbed from the intestines of either normal or X-irradiated rats; in these experiments endotoxin recovered from the gut was unaltered in its biological and serological specificity. Shreeve and Thomlinson (1972), using colostrum-deprived piglets, found by fluorescent-labelling techniques that frozen and thawed extracts of E. coli (Erskine, Sojka and Lloyd, 1957), which presumably contained endotoxin, were rapidly absorbed from the intestinal tract and were detected in the spleen; symptoms of anaphylactic shock developed thereafter.

In order to assess the significance of these findings it would be necessary to analyse the material prepared by Shreeve and Thomlinson for endotoxin and if present to isolate this component.

Investigations concerned with the cause of 'oedema disease' of swine raises the problem of the role of E. coli endotoxin in enteric infection. 'Oedema disease', unlike diarrhoeal disease, is considered to be a generalised toxaemia resulting from the proliferation of certain specific serotypes of E. coli in the small intestine and absorption of toxic components (Sojka, 1965; Nielsen, Moon and Roe, 1968). The clinical symptoms suggested a form of endotoxaemia (Thomlinson and Buxton, 1962; 1963; Nagy, Berczi and Bertok, 1968). In later studies Shreeve and Thomlinson (1970a; 1970b; 1971a) considered that the disease resulted from the development of hypersensitivity following absorption of E. coli antigens including endotoxin. Other workers (Erskine et al., 1957; Timoney, 1957; Gregory, 1960; Nielsen et al., 1965; Smith and Halls, 1968b) have suggested that a toxin distinct from endotoxin is absorbed from the intestinal tract of affected pigs.

Nielsen and Clugston (1971) attempted to show that an 'oedema-disease' principle (EDP) distinct from classical endotoxin was responsible for the symptoms of this disease. Unfortunately they administered their preparations intravenously and it is therefore difficult to draw any conclusions without knowing if absorption from the small intestine would have occurred after oral or intragastric administration of EDP. Also, it is likely that their preparations contained large amounts of endotoxin (Ørskov, 1971). Clearly preparations used in the study of enteric disease in experimental animals should be carefully analysed for the presence of LPS. The conflicting views reported in relation to 'oedema disease' result from the absence of such information.

Diarrhoeal disease is unlike 'oedema disease' in that damage is restricted to the small intestine, and symptoms of diarrhoea and dehydration are not generally accompanied by neurological and circulatory disorders (Nielsen and Clugston, 1971). The question of endotoxaemia does not therefore arise. However, it should be noted that endotoxin is capable of binding to cell surfaces (Gimbor and Rafter, 1969; Nela et al., 1970; Springer, Huprikar and Neter, 1970; Shands, 1971) and it is possible (especially in human diarrhoeal disease) that it may play a part in the adherence of E. coli cells to small intestinal epithelium.

2. Neurotoxins.

General description: The term "neurotoxin" has been ascribed to protein toxins elaborated by several gram-negative bacteria; Shigella dysenteriae (Boivin and Mesrobeanu, 1937a; 1937b; 1937c) and other Shigella species, Salmonella typhimurium, S. berlin, S. weslaco, Proteus vulgaris and E. coli (Mesrobeanu, Mesrobeanu and Mitrica, 1966). These neurotoxins cannot be regarded as true extracellular toxins. In general, autolysis of cells by heat-killing and alkaline extraction (Boivin, Delaunay and Sarciran, 1940; van Heyningen and Gladstone, 1953), or prolonged treatment with chloroform (Mesrobeanu et al., 1966; Mesrobeanu and Mesrobeanu, 1971) has been necessary to obtain good yields of these toxins. Small amounts of neurotoxin may be released by prolonged incubation (van Heyningen, 1971) but this cannot be considered as release of extracellular toxin (Raynaud and Alouf, 1971).

E. coli neurotoxin: Mesrobeanu and Mesrobeanu (1971) obtained crude preparations of E. coli neurotoxin by prolonged chloroform extraction of whole cells followed by precipitation at pH 3.5 with cold TCA (trichloracetic acid). They claimed that this procedure separated the acid insoluble neurotoxin from acid soluble endotoxin fractions. Nevertheless, in addition to a nitrogen

content of 10.5 - 13.5% they found up to 10% lipid and 12% reducing substances in their preparations. They noted that purified S. typhimurium neurotoxin had a nitrogen content of 15.5%, similar to that reported by van Heyningen and Gladstone (1953) for Shigella dysenteriae neurotoxin. However, when the lipid content of both S. typhimurium and E. coli neurotoxins was reduced by purification to approximately 1%, these preparations were as much as six times less toxic for mice. Furthermore, crude preparations of Salmonella and E. coli neurotoxins were relatively heat stable (80°C - 100°C for 1 hr) which suggests that neurotoxicity may not reside entirely in the protein component of these toxins.

Mesrobeanu and Mesrobeanu (1971) observed that E. coli neurotoxin cross-reacted antigenically with endotoxin prepared by the TCA method (Boivin et al., 1933; Boivin and Mesrobeanu, 1935; Boivin, 1940) but not with endotoxin isolated by the warm phenol-water method (Westphal and Jann, 1965). This raises the question of whether E. coli neurotoxin and indeed, other neurotoxins represent, at least in part, classical Boivin endotoxin since many of the clinical symptoms and pharmacological properties are similar. Immunological cross-reactions with endotoxin have been shown for extracted protein-containing toxins from Vibrio cholerae (Gallut and Grabar, 1945; Jenkin and Rowley, 1959), Shigella dysenteriae (Boroff, 1949; Boroff and Macri, 1949; Engely, 1952), S. typhimurium (Mesrobeanu and Mesrobeanu, 1971), Proteus mirabilis (Izdebska-Szymona, 1971) and E. coli (Mesrobeanu and Mesrobeanu, 1971). It has been suggested (Mesrobeanu et al., 1963; 1966) that neurotoxin may represent either the lipoprotein or polypeptide fraction of TCA-extracted endotoxin. It is noteworthy however, that no success has been achieved in complete separation of the protein from the lipid component. The relative differences observed between E. coli neurotoxin and endotoxin with regard to toxicity, chemical composition and

immunochemistry (Mesrobeanu and Mesrobeanu, 1971) may reflect variation in amounts of either neurotoxic protein or endotoxic lipid. These workers did observe that extracted neurotoxins from urinary-tract isolates of E. coli were more toxic for mice than those isolated from cases of infantile diarrhoea, a finding which suggests that neurotoxin does not play an important part in enteric infection. Its role in generalised toxaemia must await more precise characterisation of the toxic components in protein-LPS complexes.

3. Enterotoxins.

Emergence of E. coli enterotoxins: Certain serological groups and types of E. coli have been isolated on numerous occasions from enteric infections of man (see Table 3, page 7) and animals. However, the mere isolation of particular serotypes does not show a causal relationship.

In the past five years the ability of certain serogroups of E. coli isolated from man and animals to produce substances acting locally on the small intestine (enterotoxins) has been well established. Early work on entero-toxins elaborated by E. coli followed the introduction (De and Chatterje, 1953) of the ligated intestinal loop test in rabbits to study the enteropathogenicity of Vibrio cholerae. Later studies (De, Ghose and Sen, 1960; De, Ghose and Chandra, 1962) revealed that dilatation of rabbit intestinal loops was due to a thermolabile cell-free enterotoxin present in filtrates of V. cholerae cultures (for recent reviews on Vibrio enterotoxins, see Craig, 1971; 1972; Pierce, Greenough and Carpenter, 1971; Finkelstein, 1972).

Working with enteropathogenic strains, De, Bhattacharya and Sarkar, (1956), Taylor, Maltby and Payne, (1958) and Taylor, Wilkins and Payne, (1961) found that living E. coli cells were required for enterotoxicity in the ligated loop test. This was confirmed by Smith and Halls (1967b) and Gyles and

Barnum (1969) who also demonstrated a host species specificity for the ligated intestinal loop test. Thus; E. coli isolated from pigs affected pig, calf and lamb intestine; those from calves affected calf and lamb intestine; those from humans affected only rabbit intestines. Important factors in standardising this test for E. coli strains of human origin include the choice of rabbit strain, the suspending fluid for the inoculum and the age of the culture (Taylor et al., 1961).

The first suggestion of enterotoxic activity in preparations of killed organisms was by Taylor and Bettleheim (1966) who reported that chloroform-killed suspensions of E. coli, isolated from cases of infantile diarrhoea, caused dilatation of rabbit intestinal loops. However, they were unable to separate this dilating factor from the chloroform. Indeed, it has since been shown that chloroform-containing preparations which were free from bacteria or their products, caused dilatation in pig intestines (Smith and Halls, 1967c; Gyles and Barnum, 1969).

Physical forms of E. coli enterotoxin: Attempts to define the pathogenesis of E. coli enteric infections in man and animals has led to the isolation and characterization of two forms of enterotoxin, one heat-stable at 100°C (Smith and Halls, 1967c; Kohler, 1968; Kohler and Cross, 1969) and the other heat-labile at 60°C (Gyles and Barnum, 1969; Moon et al., 1970; Kohler, 1971b). The heat-stable (ST) enterotoxin appeared in supernatants of E. coli strains grown in soft agar cultures, peptone dialysate broth, or a synthetic-casamino acids medium (syncase broth, cf. Finkelstein et al., 1966). Cell free filtrates of these supernatants were shown to cause fluid accumulation and distention of ligated intestinal loops in pigs and diarrhoea lasting 3 - 10 hr after oral administration to conventional and gnotobiotic pigs. Similarly, heat-labile (LT) enterotoxin, present in bacteria-free filtrates of whole cell lysates, caused fluid accumulation and dilatation in ligated intestinal segments in pigs

and rabbits and marked diarrhoea and dehydration after intragastric administration.

The extent to which LT is present in culture supernatants is disputed. Moon et al., (1970) reported LT in culture supernatants of one strain isolated from pigs, but Kohler (1971a) observed LT activity only in cell lysates with the same strain. Gyles and Barnum (1969) found LT activity in both broth supernatants and whole cell lysates of porcine enteropathogenic E. coli. These workers observed that extracted endotoxin, capsular polysaccharide and K88 antigen from strains isolated from normal and infected pigs did not cause distention of intestinal loops.

There is also disagreement about the form of enterotoxin produced by different strains. Smith and Halls (1967c) noted that all enteropathogenic pig and calf strains of E. coli which they examined produced ST. Truszczynski and Pilaszek (1969) failed to confirm this marked correlation and Gyles and Barnum (1969) found that all but one of their enteropathogenic strains produced the LT form of toxin. By contrast, Kohler (1971b) showed that, in general, certain serogroups (e.g. 09) elaborate ST while others (e.g. 08) produce LT; only one strain, serotype 08:K87, K88 ab; H19, produced both ST and LT.

The general properties of the two forms of enterotoxin isolated from porcine E. coli strains suggest that they are different. The characteristics of ST and LT, as revealed by various treatments, are presented in Table 4. That neither form of enterotoxin was inactivated by trypsin suggests either that the active portion of both forms is not protein or is inaccessible to proteolytic digestion. The apparent conflict regarding dialysability, ultrafiltration and gel filtration of ST is unresolved, but may be due to different media, cultural conditions and harvesting techniques reported by these workers.

Table 4 : Physico-chemical characteristics of ST and LT from porcine enteropathogenic E. coli

Treatment[a.]	Effect on Enterotoxin Form	
	ST	LT
Heat: 30 min at 60°C	No effect[1-3c.]	Inactivated[4-6]
100°C	No effect[1-3]	Inactivated[6]
	Partially destroyed[7]	
pH: 1.0 - 3.0 for 18 hr.[6]	No effect	Inactivated
11.5 - 12.0 for 18 hr.[6]	Inactivated	Inactivated
Incubation with trypsin[6]	No effect	No effect
Precipitation with:		
50% and 100% $(NH_4)_2SO_4$	Not tested	Precipitates[4]
Methanol	Does not precipitate[6]	Precipitates[6]
Ethanol or acetone	Precipitates[6]	Not tested
Extraction with		
Chloroform or butanol	Insoluble[6]	Not tested
Dialysis:	Partially diffusable[2]	Non-diffusable[4,5]
	Non-diffusable[1]	
Filtration through:		
'Diaflo' membranes of M.W. exclusion;[b.]		
less than 1,000	Some activity found in filtrate[8]	Not tested
less than 10,000	Most of activity found in filtrate[8]	Not tested
greater than 10,000	Some activity found in filtrate[8]	Not tested
Sephadex G - 15	Eluted after sucrose[6] (M.W. less than 342)	Not tested
G - 200	Not tested	Eluted with void volume[9] (M.W. at least 2 x 10^5)

a. After treatment preparations were tested for enterotoxic activity

b. ST isolated from a calf strain with similar properties to ST isolated from porcine strains

c. Authors: 1. Smith and Halls (1967c). 2. Kohler (1968). 3. Kohler and Cross (1969).
4. Gyles and Barnum (1969). 5. Moon et al., (1970). 6. Kohler (1971b).
7. Truszczynski and Pilasczek (1969). 8. Bywater (1972).
9. Engstrom et al., (1969; cf. Kohler, 1971b).

Recent studies (Sack et al., 1971; Gorbach et al., 1971) have shown that human enteropathogenic strains of E. coli isolated from cases of acute cholera-like diarrhoea in India elaborate LT similar to that described by Gyles and Barnum (1969) from porcine isolates. In addition, E. coli isolates from human diarrhoeal disease in India (Etkin and Gorbach, 1971) and America (Gorbach, 1971 cf., Craig, 1971) which were not commonly considered as enteropathogenic, produced both ST and LT. The toxins isolated from American strains differed in potency from the Indian strains when tested in suckling rabbits (Dutta and Habba, 1955). This suggests either that LT exists in more than one form or that it is produced in different amounts by different strains of E. coli. From this brief account it is clear that the physico-chemical characterisation of E. coli enterotoxin is very incomplete.

Antigenicity of enterotoxin: Several findings show that ST is non-antigenic. Suckling pigs from sows immunised with either viable organisms or ST preparations showed no protection when challenged intragastrically with these agents (Kohler and Bohl, 1966; Kohler, 1971b). Also, ST was not neutralised by rabbit antiserum prepared against viable cells of the ST-producing strain (Gyles and Barnum, 1969). The findings of other workers (Smith and Gyles, 1970; Wilson and Svendsen, 1970; cf., Gyles, 1971) also point to the non-antigenicity of ST; they showed that ST was not neutralised by antiserum to itself or by antiserum prepared from homologous or heterologous serotypes which produce either ST or LT.

By contrast, available evidence suggests that LT is antigenic. Gyles and Barnum (1969) found that rabbit antiserum to LT neutralised the enterotoxic effect of LT from the same or other strains of E. coli in ligated intestinal loops of pigs. Antiserum against non-enteropathogenic strains did not neutralise LT. They also observed reciprocal cross-neutralisation

between V. cholerae and its enterotoxin and whole cell lysates (LT) or viable E. coli 08:K87, K88ab:H19 isolated from pigs. Smith and Gyles (1970) noted further that neutralising antibody to LT activity was induced in pigs injected intravenously with living cultures of E. coli strains which produced either LT and ST or only the ST form. They suggested however that LT activity is probably present in ST-producing cultures but in quantities too small for detection by the ligated loop test.

The ability of anti-LT serum to protect against LT activity in gnotobiotic pigs depends on how antiserum is administered (Miniats, Mitchell and Barnum, 1970; Kohler and Cross, 1971). Antiserum to LT mixed with milk and fed for 3 hr before and 9 hr after administration of LT was protective whereas intraperitoneal injection of LT-antibody 16 hr before feeding LT orally did not induce protection. Very recent studies (Smith and Linggood, 1971a; Shreeve and Thomlinson, 1971b; Wilson, 1972; Rutter and Anderson, 1972; Smith, 1972a; 1972b) showed that antibody prepared against strains of E. coli which produce LT may be important in preventing experimental oral infection with these strains.

Biological properties: As previously mentioned, both ST and LT cause distention and fluid accumulation in ligated intestinal loops of susceptible animals and produce diarrhoea after oral or intragastric administration. As in cholera (Gangarosa et al., 1960; Elliot et al., 1970) the experimental infection with toxigenic E. coli ST or LT produces no histological lesions in small intestinal epithelium (Smith and Jones, 1963; Moon, Sorensen and Sautter, 1966; Kohler, 1967; 1971b; Kohler and Cross, 1969; Bohl and Cross, 1971).

Similarities between choleric diarrhoea and enteric infections caused by enteropathogenic E. coli have led to the demonstration that their respective heat-labile enterotoxins have many biological properties in common (Table 5). The only major difference is that E. coli enterotoxin is not

Table 5: Biological properties of heat-labile enterotoxins from E. coli and V. cholerae

Property	Reaction of Enterotoxin From: E. coli	V. cholerae[1.]
Inhibition of net sodium absorption	Yes[2.]	Yes
Inhibition of glucose absorption	No[1.]	No
Net chloride secretion	Yes[2.]	Yes
Increased adenyl-cyclase activity	Yes[1.]	Yes
Increased lipase activity in rat fat cells	Yes[1.]	Yes
Inactivation by ganglioside	No[1.]	Yes
Reactivity after intravenous injection : diarrhoea	No[3.]	No
lethality	?	Yes (mouse, dog)
Increased vascular permeability (rabbits, guinea pigs)	Yes[4.]	Yes
Visible swelling of rat feet	Yes[4.]	Yes

1. Craig (1971). 2. Al-Awqati, Wallace and Greenough (1972). 3. Kohler (1968).

4. Moon and Whip (1971).

inactivated by ganglioside. This suggests that although both enterotoxins appear to affect the same processes in the small intestine, they may have different binding sites in epithelial mucosa.

Role of enterotoxins in E. coli pathogenicity: Studies on cholera enterotoxin have demonstrated quite convincingly that the production of diarrhoea is due to the local activity of a single antigenic, enterotoxic moiety on ion transport processes in the small intestine. The situation with regard to E. coli enterotoxins appears to be less well defined. Certainly, the majority of enteropathogenic E. coli isolated from swine elaborate a heat-labile enterotoxin which has many properties in common with cholera toxin. However, some of these strains also produce a heat-stable enterotoxin which is not neutralised by anti-enterotoxin sera and shows no cross reactivity with cholera enterotoxin. The significance of the heat stable enterotoxin in enteric infection is not well understood.

Also it should be emphasised that enterotoxins isolated from human enteropathogenic strains of E. coli do not cross react with cholera enterotoxin (Sack et al., 1971). In addition, LT from human isolates was poorly antigenic (Etkin and Gorbach, 1971) and it was suggested that recurrent episodes of "non-specific diarrhoea" caused by toxigenic E. coli (Gorbach, 1970; Etkin and Gorbach, 1971) might be due to poor convalescent antitoxin levels. Indeed, enough evidence now exists to permit the suggestion that LT from human strains is not identical with LT from porcine isolates.

A further point regarding E. coli enterotoxins from human and animal strains is worth noting. It has been demonstrated (Smith and Halls, 1968a; Smith and Gyles, 1970) that the production of both LT and ST activity from porcine strains is controlled by a plasmid which can transfer the property to other strains of E. coli and certain Salmonella species. Smith and Linggood,

(1971b), found however, that without the co-transfer of K88 antigen no diarrhoea developed in pigs fed orally with these strains. As pointed out previously, although many enteropathogenic E. coli of porcine origin possess K88 antigen, this factor is not common to human enteropathogenic strains. In tests with human isolates of E. coli from cases of diarrhoea, Smith and Linggood (1971a) were able only to transfer the enterotoxin plasmid from one of 27 strains to a recipient E. coli K12F⁻ strain. This situation may be somewhat analagous to the normal low frequency of transfer of drug-resistance factors (Datta, Lawn and Meynell, 1966) and may account for the infrequent appearance of new serotypes of toxigenic E. coli (Ørskov et al., 1960; Etkin and Gorbach, 1971; Love et al., 1972) which are not of the commonly accepted enteropathogenic types.

Thus, clear differences are emerging between human and animal strains of enteropathogenic E. coli. They produce similar diseases in their respective hosts, and produce enterotoxins which appear to be responsible for the symptoms of diarrhoea and dehydration. However, the mechanisms of initiation of infection seem to be different.

4. Haemolytic activities of E. coli.

Attempts to correlate haemolytic activity with pathogenicity: It is well established that some strains of E. coli isolated from various sources produce zones of haemolysis when plated on blood agar medium. However, most authors have merely noted in passing that haemolytic E. coli were isolated and few attempts have been made to correlate this property with the pathogenicity of the organism.

Early studies (Dudgeon et al., 1921; 1922; Meyer and Löwenberg, 1924) suggested an epidemiological association of haemolytic Bacterium coli with acute urinary-tract infections and diarrhoeal disease. The strains isolated appeared to be closely related antigenically. However, such early studies

must be interpreted with caution since it is generally agreed that serological analysis of E. coli was unreliable prior to the extensive investigations of the Scandinavian workers in the 1940's (see page 27).

Sjöstedt (1946) showed that the haemolytic capacity of E. coli was related to its virulence in mice. Of 56 serological types, graded according to the response of mice challenged with whole cells, 14 (24%) were haemolytic and 13 of these types included strains which were rapidly lethal for mice. Analysis of strains showed that 62 (79%) of 78 haemolytic strains were necrotising, whereas only 125 (27%) of 464 non-haemolytic strains caused necrosis in mouse skin. Furthermore, of the non-necrotising strains, only 4.5% were haemolytic, whereas 33% of necrotising strains were haemolytic. Kauffmann (1948) confirmed the necrotising activity of haemolytic E. coli.

That dermonecrosis could be produced in guinea pigs and rabbits with supernatant fluids of centrifuged broth cultures of E. coli was demonstrated by Smith (1963). He noted however, that membrane filtration of these fluids destroyed the necrotising capacity. His explanation for the loss in reactivity was a reduced content of haemolytic activity in the filtered preparations. It seems equally likely that the necrotic activity of supernatant fluids was due to viable organisms. Indeed, no investigators have demonstrated conclusively that the haemolytic principle of E. coli is either dermonecrotic or lethal. Studies which have employed whole cells or crude supernatant preparations must be considered with caution.

In contrast with E. coli enterotoxins which have been intensively studied lately, few attempts have been made to characterise the haemolytic activities of E. coli or to determine the role of haemolysin in pathogenicity. Lack of knowledge in this field was the stimulus for the present study.

Production of extracellular, filterable haemolysin: No single strain or serotype has been employed exclusively in studies of the haemolytic activity of E. coli. A variety of E. coli isolates from calves (Lovell and Rees, 1960), pigs (Smith, 1963; Short and Kurtz, 1971; Muranyi and Juhasz, 1971) and humans (Snyder and Koch, 1966) have been used for the production of filterable haemolysin (designated α-haemolysin by Smith, 1963). This makes the task of comparing results reported by different workers difficult.

A variety of different media have also been employed in attempts to demonstrate extracellular haemolytic activity. Early workers (Kayser, 1903; Dudgeon et al., 1921; 1922; Dudgeon and Pulvertaft, 1927; Sjöstedt, 1946; Kauffmann, 1951; Robinson, 1951; Bamforth and Dudgeon, 1952; Duguid et al., 1955; Ishii, 1960) usually employed peptone-water medium, to which was added sodium chloride, glucose, serum, or combinations of these ingredients, yet none of these workers were able conclusively to demonstrate α-haemolytic activity. Indeed Robinson (1951), in attempts to repeat the work of Kayser (1903) concluded that the filterable haemolytic principle reported by this author was simply carbonate.

Using a glucose-nutrient broth medium to which was added an alkaline extract of veal, Lovell and Rees (1960) and Smith (1963) were the first workers able clearly to demonstrate α-haemolytic activity in culture filtrates. More recently, commercially prepared beef- and brain-heart infusion media, and acid hydrolysates of casein have been used to produce α-haemolysin (Snyder and Koch, 1966; Short and Kurtz, 1971). In addition, production of filterable haemolysin has been reported (Snyder and Koch, 1966) and confirmed (Short and Kurtz, 1971) by a strain of serogroup O6 in a chemically defined medium containing glucose (0.2% w/v) as carbon source. Filterable activity was not produced by a different strain in a similar medium in which glucose was substituted by lactose (Robinson, 1951). In a recent investigation (Muranyi

and Juhasz, 1971) filterable haemolytic activity elaborated by porcine strains of E. coli in the chemically defined medium of Snyder and Koch (1966), occurred only if the pH of the culture fluid fell below 5.4. These workers concluded that previous demonstrations of α-haemolysin were due to the production of organic acids, and especially lactic acid.

Examination of haemolysin production reported by Snyder and Koch (1966) shows that filterable haemolytic activity reached a maximum at pH 5.2 - 5.4. Since haemolytic activity in culture filtrates of E. coli grown in chemically defined medium is heat resistant (Snyder and Koch, 1966; Muranyi and Juhasz, 1971) the possibility that organic acids are responsible for haemolysis cannot be discounted.

In unshaken cultures (alkaline extract of veal), maximum levels of α-haemolysin appeared within 4 hr of incubation (Lovell and Rees, 1960; Smith, 1963). In addition, Smith (1963) showed that α-haemolysin appeared much earlier (1 - 2 hr) in the growth cycle if the inoculum size was increased 100-fold. By contrast, Snyder and Koch (1966) who used beef-heart infusion broth and Short and Kurtz (1971) who employed the medium of Smith (1963), observed maximum α-haemolysin production in shake cultures at the onset of the stationary phase of growth. A summary of reported cultural conditions and their effect on α-haemolysin production is presented in Table 6. It is quite likely that disagreement on the effect of carbon dioxide may reflect strain variation. Of particular interest is the observation that large molecular weight substances contained in peptone, milk and ovalbumin enhance the production of α-haemolysin. Inukai and Kodama (1965) have suggested that non-dialysable factors are related to the release of α-haemolysin from the cell rather than to its synthesis.

Haemolytic activity associated with whole cells (designated β-haemolysin by Smith, 1963) is often produced in conjunction with extracellular

Table 6: Influence of cultural conditions on production of *E. coli* α-haemolysin

Medium	Condition Varied	Effect on α-Haemolysin Production
Complex	Speed of Shaking	Shaken cultures showed higher activities than unshaken cultures.[1.*]
Complex	Gas Phase	Haemolysin production better in air than in 20% CO_2 or under anaerobic conditions[1.]; CO_2 and H_2 had no effect on production.[3.]
Complex	Temperature	Production optimal at temperatures between 30°C and 40°C.[4.]
Complex	Carbohydrate	Production not influenced by adding 7 different sugars to casein hydrolysate medium.[5.]
Defined	Carbohydrate	Of 7 sugars added at 0.2% (w/v) concentration only glucose, lactose and mannitol supported production.[5.]
Complex	Nitrogen Source	Large molecular weight components of peptone, milk and ovalbumin enhanced production.[2,6.]
Complex	Nitrogen Source	Peptone, on removal of large molecular weight components failed to support production.[2.]
Complex	Lipids	No haemolysin produced in a medium composed of lipids extracted from alkaline extract of veal.[2.]

* Authors. 1. Snyder and Zwadyk (1969). 2. Short and Kurtz (1971). 3. Lovell and Rees (1960).
4. Smith (1963). 5. Snyder and Koch (1966). 6. Inukai and Kodama (1965).

α-haemolysin (Smith, 1963). Clearly, separation of the haemolytic activities of this organism requires the use of filters which retain whole cells yet allow the passage of extracellular haemolysin. A variety of filters have been employed for this purpose and are summarised in Table 7. Difficulties experienced by workers prior to 1960 in demonstrating α-haemolytic activity might be related to the type of filter used. However, there has been no systematic examination of the adsorption or inactivation of α-haemolysin by different filters.

Studies on cell-associated (β-) haemolysin: Few attempts have been made to determine the relationship of β-haemolytic activity to the extracellular α-haemolysin. Most workers agree that β-haemolytic activity requires the presence of viable, metabolising cells. Indeed, Smith (1963) found that mechanical disruption of cells or addition of streptomycin abolished only β-haemolytic activity. Also antiserum to α-haemolysin did not neutralise β-haemolysin. However, various erythrocyte species were equally sensitive to α- and β-haemolysins. Snyder and Koch (1966) observed that both α- and β-haemolysin produced in meat infusion broth were heat-labile ($56^{\circ}C$ - 1 hr) and sensitive to 1% formaldehyde (v/v).

A close association between β-haemolysin and viable cells is supported by the studies of Short and Kurtz (1971). They were unable to dissociate β-haemolytic activity from whole cells of E. coli 0141 by sonic treatment, freezing and thawing, autolysis or digestion with trypsin or lysozyme. Furthermore, they noted that cell-associated activity decreased as the number of viable cells decreased and that any agents, such as potassium cyanide, streptomycin, rifamycin and nalidixic acid, which affected cellular metabolism, completely inhibited β-haemolytic activity but did not affect α-haemolysin. When viable E. coli cells containing β-haemolysin were separated from a suspension of sheep erythrocytes by a Visking dialysis

Table 7 : Filtration of *E. coli* supernatants containing haemolytic activity

Type of Filter	α-Haemolytic Activity[1.]	Author
Berkefeld M. Candle	-	Dudgeon and Pulvertaft (1927); Bamforth (1944 cf. Bamforth and Dudgeon, 1952)
Sintered Glass	-	Bamforth (1944 cf. Bamforth and and Dudgeon, 1952)
	±	Ishii (1960)
Gradacol	-	Bamforth and Dudgeon (1952)
Seitz	-	Bamforth (1944 cf.)
Chamberland	-	Ishii (1960)
Ford SD Sterimat	+	Lovell and Rees (1960); Smith (1963)
Millipore (Cellulose-Ester)	+	Snyder and Koch (1966); Short and Kurtz (1971)

1. + 50 - 100% of supernatant activity
   ± 10 - 50% of supernatant activity
   - No activity in filtrates

membrane, no haemolysis occurred. This indicated that the β-haemolysin was not a diffusible metabolite of E. coli.

Although the α- and β-haemolysins differ in their association with the cell surface, both require divalent cations for activation (Robinson, 1951; Bamforth and Dudgeon, 1952; Snyder and Zwadyk, 1969), and it is possible that α-haemolysin is a cell-free form of the β-haemolysin. If this is the case, the failure of anti-α-haemolysin to neutralise β-haemolytic activity (Smith, 1963) can be interpreted to mean that the antibody-combining site of β-haemolysin is inaccessible to anti-α-antibody. Alternatively this finding may indicate that the α- and β-haemolysins are not localized in the same moiety.

Gamma (γ-) haemolysin: Mutant strains of E. coli, resistant to nalidixic acid, have been shown to produce a "diffusible" haemolysin (designated γ-) which may be different from α-haemolysin (Walton and Smith, 1969). No activity was observed against rabbit or human erythrocytes whereas α-haemolysin was active against all erythrocyte species tested. These experiments were carried out only on indicator agar and physico-chemical characteristics of this haemolytic principle were not investigated. To my knowledge no further studies have been made on γ-haemolysin.

Purification of α-haemolysin: Only two authors have attempted to purify the extracellular haemolysin elaborated by E. coli. The reasons appear to be two-fold; firstly, most workers have been unable to obtain high yields of α-haemolysin in crude filtrates, and secondly, α-haemolysin seems to be unstable (see page 29).

Zwadyk and Snyder (1971) reported a 23-fold increase in specific activity by precipitating the haemolysin with ethanol (25% v/v) at pH 4.5 in

the cold. The precipitate which formed was re-dissolved in 0.15M NaCl. Ammonium sulphate was then added to 30% (w/v) saturation and the precipitate was again re-dissolved in 0.15M NaCl. However, like Lovell and Rees (1960), Zwadyk and Snyder noted that removal of ammonium sulphate by dialysis resulted in loss of haemolytic activity. They suggested that the haemolysin, at least in part, might be a small molecular weight substance but they did not attempt to mix the diffusate with the dialysate to substantiate their suggestion. The findings of Short and Kurtz (1971) do not support the idea that the α-haemolysin has a small molecular weight. These workers found that chromatography of crude α-haemolysin preparations on Sephadex G-200 and Sepharose 6B resulted in the appearance of two closely associated, large molecular weight peaks of activity. Approximately 80% of the total activity was recovered. Only one peak of activity was observed after fractionation on DEAE (diethylaminoethyl) cellulose. No indication of the extent of purification was given and furthermore, the activity of the starting material was very low.

In view of the discrepancies between the findings of Zwadyk and Snyder (1971) and Short and Kurtz (1971) regarding molecular weight and since neither group succeeded in producing high titre purified toxin, purification of E. coli haemolysin was a major aim of this thesis.

The nature of E. coli α-haemolysin: Most studies with E. coli α-haemolysin have been performed with crude or partially-purified preparations. Several findings however, have given an indication of the physico-chemical nature of this haemolytic principle; these are recorded in Table 8. It can be seen that α-haemolysin is heat-labile, although inactivation at 37°C could be retarded if the pH of filtrates was adjusted to 3.0 (Smith, 1963). Treatment with proteolytic enzymes in addition to chemical analysis are consistent with the view that α-haemolysin is a protein. The dispute regarding the molecular size of α-haemolysin has been mentioned above.

Table 8 : Physico-chemical characteristics of *E. coli* α-haemolysin

Treatment	Effect on α-Haemolysin
Heat	Total inactivation in 10 min at 56°C and in 24 hr at 37°C.[1.*]
Storage	At -20°C, all activity retained for more than 10 days; 50% of activity lost after 4 days at 5°C.[1.]
Addition of Metal Ions	Activated by calcium and strontium ions; 5 mM optimal:[2.] 10 mM calcium chloride optimal.[3.]
Proteolytic Enzymes	Inactivated by trypsin and chymotrypsin.[2.]
Other Enzymes	Not inactivated by DNase, RNase, lecithinase or lysozyme.[2.]
EDTA	Inactivated if calcium ions present.[3.]
Lipids	Not inactivated by lecithin or cholesterol.[3.]
Reducing Agents	Not activated by mercaptoethanol, ascorbic acid or dithiothreitol.[3.]
Chemical Analysis	Only protein found; no carbohydrate or lipid.[4.]
Dialysis	Activity lost, i.e. molecular weight less than 12,000.[4,5.]
Gel Filtration	Eluted with void volume of Sephadex G-100, i.e. molecular weight greater than 1×10^5.[3.]

* Authors. 1. Smith (1963). 2. Snyder and Zwadyk (1969). 3. Short and Kurtz (1971).
4. Zwadyk and Snyder (1971). 5. Lovell and Rees (1960).

Reference to Table 8 shows that α-haemolysin requires divalent cations for activation. By exhaustive dialysis of veal infusion broth before inoculation with E. coli Snyder and Zwadyk (1969) demonstrated that calcium ions were required only for activation, not for production.

More precise studies have suggested that a complex, consisting of haemolysin, calcium and erythrocytes, must be maintained up to the lytic event (Short and Kurtz, 1971). These authors noted that addition of EDTA at any time during haemolysis inhibited the reaction. It is also notable that reducing agents had no effect on haemolysis indicating that -SH groups are probably not involved in the interaction of the haemolysin with erythrocytes. Since the properties of limited amounts of purified haemolysin have been studied by only two groups, information on important aspects is fragmentary. Also, the experiments of Zwadyk and Snyder (1971) and Short and Kurtz (1971) are open to criticism. Both groups used methods which involved the centrifugation of haemolysin-erythrocyte mixtures at different times, followed by spectrophotometric estimation of haemoglobin; this inevitably involves a delay during which haemolysin-affected cells may be lysed. In the present study the turbidity of reaction mixtures was monitored continuously at 650 nm, allowing rate measurements to be made on haemolysis curves.

A study of the biological properties of E. coli α-haemolysin has been performed by only a single author (Smith, 1963). Therefore, no comparisons can be made of the biological activities of different α-haemolysin preparations.

Smith found that 0.4 ml volumes of crude supernatant fluids or filtrates, injected intravenously, were required to kill 40% of mice injected. Similarly, large volumes of these preparations were necessary to kill rabbits and guinea pigs. Supernatant fluids were on occasion more active and,

although the contribution of organisms in these fluids cannot be excluded, supernatants from β-haemolytic and non-haemolytic cultures never caused death.

Intragastric administration of doses of α-haemolysin, considered lethal by the intravenous route, caused no adverse effects in rabbits, mice and guinea pigs. In addition, Smith found that antibody to crude α-haemolysin, produced in rabbits, neutralised zones of haemolysis from strains which produced α-haemolysin but not from strains which produced only β-haemolysin. He suggested that the antibody was protective since mice injected with sub-lethal doses of α-haemolysin were protected against subsequent challenge 8 days later with a lethal amount of culture filtrate. No protection against challenge with active haemolysin was observed if mice were injected with α-haemolysin which had been stored for 24 hr at 37°C. Pre-injection with heat-killed E. coli cells also failed to protect against the lethal effect of α-haemolysin.

Smith was only able to demonstrate a dermonecrotic response in 33% of rabbits and guinea pigs using supernatant fluids which contained α-haemolytic activity. The dermonecrotic activity was lost after filtration through membrane filters although hard swellings appeared at the site at which filtrate was injected.

The effect on tissue culture cells of culture filtrates containing α-haemolytic activity has been investigated by Chaturvedi et al., (1969). A cytopathic effect was produced in chick embryo cells but not in monkey kidney or mouse embryo cells. However, due to differences in heat stability and neutralisation properties, these authors concluded that α-haemolysin and cytotoxic factor were not identical.

Role of haemolysins in enteropathogenicity of E. coli: The role of the haemolytic activities of E. coli in enteric disease is not known. There is a divergence of opinion among the few authors who have investigated this

property of E. coli as to conditions necessary for in vitro production of extracellular haemolysin. Only limited attempts have been made to purify and characterise the extracellular haemolysin and study its biological effects and mode of action.

Like enterotoxin and K88 antigen, the production of α-haemolysin is controlled in a minority of strains by a transmissible plasmid (designated Hly, Smith and Halls, 1967a), but the significance of this function in enteric disease is not clear. Smith and Linggood (1971b) did observe that porcine isolates of E. coli which carried the Hly plasmid were more virulent for mice but they were unable to correlate this property with the production of diarrhoea or dilatation of ligated intestinal loops in experimentally infected animals.

Smith and Halls (1968b) have suggested that α-haemolysin might have a function in bowel 'oedema disease' of swine. It has been shown that heated supernatants of intestinal fluids from affected pigs no longer produce the symptoms of 'oedema disease' (Lloyd, 1957; Timoney, 1957). Furthermore, it is thought unlikely that enterotoxin is absorbed from the intestinal tract and some of the serotypes of E. coli, such as O139:K82, commonly associated with 'oedema disease', do not produce enterotoxin (Smith and Halls, 1967a). Since α-haemolysin is heat-labile (Table 8) and is almost invariably produced by E. coli isolated from cases of bowel oedema (Smith and Halls, 1968b) these authors were prompted to speculate on the role of α-haemolysin in 'oedema disease'. They concluded that a direct toxic effect was unlikely because serum from most pigs has a reasonably high anti-α-haemolysin content (Smith, 1963) and intravascular haemolysis is not a common feature of the condition. No experimental evidence has been presented to confirm or disprove this suggestion. No authors have investigated the effects of E. coli α-haemolysin in ligated intestinal loops and only Smith (1963) has administered α-haemolytic

filtrates intragastrically to laboratory animals. The function of the haemolytic activities of E. coli in human enteric infection is even more obscure.

In my opinion, there have been so few investigations of the haemolytic activities of E. coli that it is impossible to evaluate, at present, the role, if any, of this agent in pathogenicity. This will be possible only after much more is known of the nature and biological properties of E. coli haemolysins.

MATERIALS AND METHODS

MATERIALS AND METHODS

A. Survey of the Haemolytic Activity of E. coli.

1. Strains of E. coli.

The strains of E. coli used in this investigation were obtained through the courtesy of Dr. T.A. McAllister, Consultant Bacteriologist, Queen Mother's Hospital, Yorkhill, Glasgow. Isolates were received on nutrient agar slopes and had been recovered initially from faeces, urines and miscellaneous infections from hospitalised individuals and outpatients. Several serologically typed strains, isolated originally from diagnosed cases of human gastroenteritis were received in lyophilised ampoules from the National Collection of Type Cultures (NCTC), Colindale, London. These were:

1. NCTC 8003; 0124:K72 (B17):H30
2. NCTC 8007; 0111:K58 (B4) :H2
3. NCTC 8009; 0111:K58 (B4) :H2
4. NCTC 8179; 0111:K58 (B4) :H2
5. NCTC 8603; 055 :K59 (B5) :H6
6. NCTC 8620; 026 :K60 (B6) :H-
7. NCTC 8621; 086 :K61 (B7) :H-
8. NCTC 8622; 0126:K71 (B16):H2
9. NCTC 8623; 0125:K70 (B15):H19

2. Checks for purity and maintenance of cultures.

On receipt, all cultures were gram-stained and plated onto nutrient agar (Oxoid No. 2 broth + 1.5% Difco Bacto Agar) or Tryptone Soya Agar (Oxoid) and MacConkey's bile salts agar (Oxoid). Any contaminated strains or cultures with non-uniform colony size were re-plated and checked again to confirm their purity and colonial morphology.

Cultures were maintained by lyophilisation or by monthly passage on freshly prepared nutrient agar slopes. The latter method was employed with those strains which were used regularly. All strains, whether lyophilised or maintained on agar slopes stored at $4^{\circ}C$, were plated onto

nutrient agar, MacConkey's agar and sheep erythrocyte agar medium (see Appendix I) to check for contaminants and uniform colonial morphology before experimental use.

3. Plate haemolysis test.

Sheep erythrocyte overlay medium: The preparation of this medium is recorded in Appendix I. Sheep blood was collected aseptically by jugular venepuncture from black-faced mountain ewes into anticoagulant which consisted of sterile 3.8% (w/v) sodium citrate in 0.85% (w/v) saline. Blood obtained in this way was never used if more than 3 days old.

Inoculation of cultures: Several single colonies with the same morphology were transferred from nutrient agar onto sheep erythrocyte overlay medium and the plates were incubated overnight at 37°C. Haemolysis was recorded as positive (+), or negative (-).

4. Serological typing.

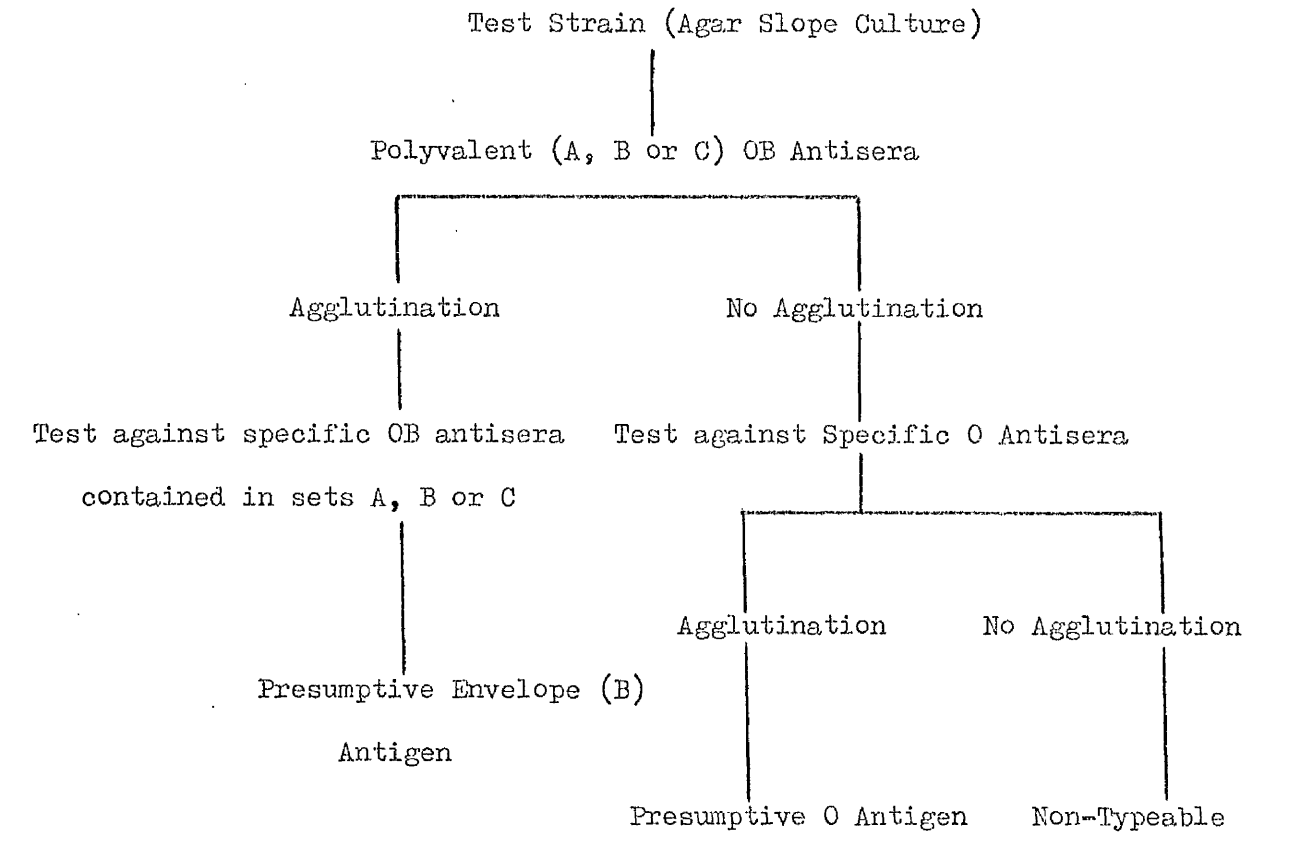
Antiserum: E. coli agglutinating antisera were obtained from Difco (Detroit, U.S.A.). Table 9 shows the antisera which were purchased.

Presumptive serological test (after Kauffmann, 1966): Presumptive analysis of envelope (B) and somatic (O) antigens was performed as outlined in Figure 1. A number of colonies from haemolytic and non-haemolytic strains of E. coli were transferred from MacConkey agar plates onto nutrient agar slopes and were incubated for 18 hr at 37°C. A loopful of growth from the slope was mixed with a drop of saline on a clean microscope slide. To this suspension was added a drop of polyvalent set A, B or C antiserum (Table 9). The slide was rocked back and forth for 1 - 2 min and the suspension was examined for agglutination. A known enteropathogenic serotype was included as a positive control; a suspension of the test strain in saline served as a control for

Table 9 : *E. coli* agglutinating antisera purchased

Antiserum Preparations	Components
Polyvalent OB Set A	026:B6; 055:B5; 0111:B4; 0127:B8
Polyvalent OB Set B	086:B7; 0119:B14; 0124:B17; 0125:B15; 0126:B16; 0128:B12
Polyvalent OB Set C	018:B21; 020:B7; 020:K84(B); 028:B18; 044:K74; 0112:B11
Specific OB Antisera	Single OB antisera of polyvalent sets A, B and C
Single O Antisera	1-19, 21-25, 45, 62, 68, 75, 102, 113, 136, 140

igure 1 : Outline of presumptive serological typing by slide agglutination



autoagglutination. If agglutination occurred in one of the polyvalent antiserum sets the test strain was examined against each of the specific OB antisera contained in the positive polyvalent set. Strains which did not agglutinate with any of the polyvalent antisera were tested against single O antisera. The high cost of agglutinating antisera prevented a more comprehensive antigenic analysis of the test strains.

B. Preparation and Harvesting of Haemolytic Activity.

1. Strain of E. coli used.

A single strain of E. coli, 25238, isolated from a midstream urine from a young girl and serotyped as E. coli O19:B7, of unknown flagellar type, was employed in experiments designed for the production of α- and β-haemolysin.

2. Media.

Chemically defined medium of Snyder and Koch (1966): The ingredients of this medium (see Appendix I) were used at the concentrations described by these authors. Only the concentration or type of carbon source when necessary was altered. This medium is hereinafter referred to as CDM.

Alkaline meat-extract broth of Lovell and Rees (1960), modified by Smith (1963): Details of the preparation of this medium are given in Appendix I. The only difference in this medium was that instead of veal, fresh beef hearts were obtained from Spence Brothers at a Glasgow slaughterhouse. This medium is referred to in the text as MEB.

Nutrient broth (Oxoid No. 2): This medium was prepared as described by the manufacturers. After autoclaving at 15 lb/in^2 for 15 min, α-D glucose (BDH-Analar), sterilised by filtration through a Millipore filter (Millipore

Corp. London) of 0.45μ porosity was added aseptically at 0.2% (w/v) final concentration. This medium is hereinafter designated as NBG.

Other media: Beef Heart for infusion (Difco), Brain Heart Infusion (Difco), yeast extract-casein hydrolysate medium (Bernheimer and Schwartz, 1963; modified by McNiven, 1972) and Tryptone Soya broth (Oxoid) were employed occasionally. These were prepared as described by the manufacturers or authors with the addition of 0.2% (w/v) glucose.

3. Preparation of α- and β-haemolysin.

α-haemolysin: Bulk preparation of extracellular α-haemolysin was achieved by transfer of single haemolytic colonies of strain 25238 to several nutrient agar slopes which contained 0.2% (w/v) glucose. After 12 - 14 hr at 37°C, growth from the slopes was washed off with sterile saline and the organisms were washed twice in sterile saline in an MSE bench centrifuge (Measuring and Scientific Instruments Ltd., Crawley, England) for 15 min at 4°C. The pellet from the final wash was resuspended in a small volume (2 - 4 ml) of sterile saline and aliquots of this suspension were inoculated aseptically into 500 ml of NBG or MEB contained in 2 litre dimpled Erlenmeyer flasks. The initial optical density of the cultures, measured at an extinction (E) of 660 nm in a Unicam SP600 spectrophotometer (Pye Unicam, Cambridge, England) with glass cells of 0.5 cm light path, was adjusted with bacteria or broth to 0.080 ± 0.01. Sterile medium was used in the reference cell.

The cultures were incubated in ar orbital shaking incubator (A. Gallenkamp & Co. Ltd., London) at 37°C for 2 - 3 hr; the shaking speed was 150 rev/min with the orbital piston stroke set at 3 cm. Thereafter the culture fluid was centrifuged at 22,000 g for 15 min at 4°C in an MSE High Speed 25 centrifuge. The supernatant fluid was decanted into a vessel pre-chilled to 4°C and was immediately passed with positive nitrogen pressure

(2 lb/in^2) through a Millipore filter of 0.45μ porosity. The culture filtrate was then titrated (page 43) and stored at -20°C until required.

The ability to obtain large yields of α-haemolysin in culture filtrates depended on these critical factors :

1. Immediate centrifugation of whole culture fluid was required since continued metabolic activity of the organisms led to the disappearance of α-haemolytic activity.

2. It was necessary to perform all harvesting procedures at 4°C to prevent multiplication of organisms which remained in supernatants even after centrifugation.

3. Use of a large (143 mm diameter) Millipore filter unit and positive pressure was required for filtration of large amounts of supernatant fluid. E. coli began to appear in culture filtrates when large volumes of supernatant were passed through smaller (43 mm) diameter filters under negative pressure.

β-haemolysin: For the preparation of large amounts of β-haemolytic activity the procedure adopted by Rennie and Arbuthnott (1971) was employed. Single haemolytic colonies of the test strain were suspended in sterile saline and transferred to agar slopes of CDM. After 12 hr incubation at 37°C growth from the slopes was washed twice in sterile saline and aliquots were inoculated into 500 ml of CDM contained in 2-litre dimpled Erlenmeyer flasks. The initial $E^{0.5\ cm}_{660\ nm}$ was adjusted to 0.040 ± 0.01 (approximately 1.5 x 10^8 cells/ml). The cultures were incubated for 3.5 hr at 37°C (stage A) in the orbital incubator at 150 rev/min when the fluid was centrifuged at 22,000 g for 15 min at 4°C. The cells were then re-suspended in sterile saline and inoculated into 490 ml of fresh CDM without glucose to an $E^{0.5\ cm}_{660\ nm}$ of 0.080 ± 0.01. After a further incubation of 3 hr (stage B) during which time no growth occurred and only small amounts of β-activity could be detected,

0.2% glucose (w/v) was added. The cells were harvested by centrifugation 2 to 3 hr later (stage C) and were titrated (Materials and Methods, Section C2) to determine their content of β-haemolysin.

4. Monitoring of growth.

Samples of culture fluid were withdrawn at 0.5 hr or 1 hr intervals and the $E^{0.5\ cm}_{660\ nm}$ was determined in the SP600 spectrophotometer. If the $E^{0.5\ cm}_{660\ nm}$ was greater than 0.40 the sample was diluted with sterile medium until the $E^{0.5\ cm}_{660\ nm}$ fell within the range 0.00 to 0.40. Sterile uninoculated medium was used in the reference cell. Growth, as $E^{0.5\ cm}_{660\ nm}$ (x dilution) was plotted on a logarithmic scale against time of sampling.

Viable cell counts were made according to the method of Miles, Misra and Irwin (1938). Serial 10-fold dilutions of culture fluid in sterile saline were prepared and 0.02 ml volumes were applied to nutrient agar plates. After overnight incubation at 37°C separate colonies were counted from 2 or 3 dilutions and the number of viable cells/ml of culture was estimated.

5. Checks for sterility.

It became apparent that viable organisms which in some way managed to pass through the Millipore filters could either contribute to or destroy α-haemolytic activity in culture filtrates. In order to ensure that the observed haemolytic activity was entirely due to α-haemolysin, immediately after passing supernatant fluids through the filter unit, samples were removed from the filtrate and were plated onto nutrient agar and MacConkey's agar medium. These plates were then examined periodically for 48 hr. If any growth, and especially lactose-positive bacteria, became evident, the filtrates were immediately re-filtered and checked again for sterility. Similar sterility checks were made during various purification procedures.

C. Haemolysin Assays.

1. α-haemolysin.

Qualitative tube titration: Serial doubling dilutions of 0.5 ml of sample filtrates were made in 0.5 ml volumes of Veronal-buffer, pH 7.3 (see Appendix II) containing 10 mM calcium chloride (V-C buffer). To each tube was added 0.5 ml of a 2% suspension of freshly collected, washed sheep erythrocytes. The tubes were incubated in a water bath for 1 hr at 37°C with periodic shaking to prevent sedimentation of unlysed erythrocytes. The titre of α-haemolysin was recorded in HU_{50}/ml as the reciprocal of the dilution which caused 50% visual haemolysis under the conditions described. This method was used when large numbers of samples were assayed to determine relative amounts of α-haemolysin present. A spectrophotometric assay, based on the amount of haemoglobin released from a standardised suspension of sheep erythrocytes, was employed for more accurate estimations of the amount of α-activity present in a particular sample.

Spectrophotometric assay of haemolysis: Sheep blood was collected as described (page 36). The blood was washed three times in sterile saline and a nominal 2% suspension was made in V-C buffer. Standardisation of this suspension was performed by lysis of a 1 ml sample of the erythrocyte suspension with a small amount of saponin. The lysed aliquot was diluted 4-fold with distilled water and the amount of haemoglobin contained in the dilution was measured in an SP600 spectrophotometer at $E^{0.5\ cm}_{545\ nm}$ in glass cells. The concentration of erythrocytes was adjusted such that 100% haemolysis of the diluted aliquot gave an $E^{0.5\ cm}_{545\ nm}$ of 0.500. The suspension was then considered to be a standardised 2% suspension of sheep erythrocytes (SRBC). Other erythrocyte species used and different concentrations of sheep erythrocytes were standardised in a similar manner.

Serial doubling dilutions of test filtrates were made in 0.5 ml volumes of V-C buffer and 0.5 ml of 2% SRBC was added to each tube. Tubes containing no haemolysin served as controls. After incubation for 1 hr at 37°C the tubes which showed approximately 50% visual haemolysis were centrifuged in an MSE Super Minor centrifuge for 2 min at 500 g. Supernatant fluid was removed with a pasteur pipette and an equal volume of distilled water was added. The 50% end point was determined by comparing the amount of haemoglobin released from the test samples with a standard for 50% haemolysis prepared by saponin lysis of a 2% SRBC suspension in the following way.

$$\frac{E_{545\ nm}\ \text{of test}}{E_{545\ nm}\ 50\%\ \text{standard}} \times \text{dilution} = \text{haemolytic units}$$

One haemolytic unit (HU/ml) was then defined as the reciprocal of the dilution which caused 50% haemolysis under the conditions of the assay.

Such values agreed well with those obtained using the gradient dilution method of Bernheimer and Schwartz, 1963 (modified by McNiven, 1972).

2. β-haemolysin.

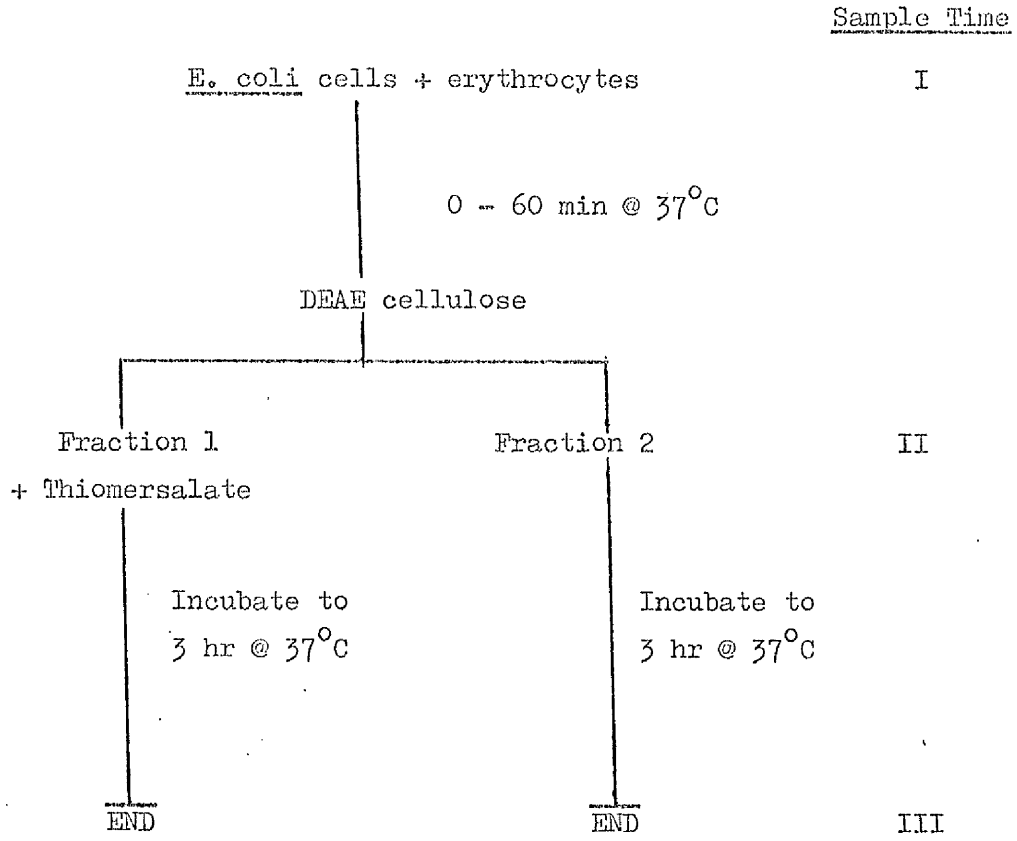
Tube titration: Cell pellets, obtained by centrifugation of whole culture fluid, were resuspended to the original culture fluid volume with V-C buffer. Doubling dilutions of aliquots of these samples were made in 0.5 ml volumes of V-C diluent and 0.5 ml of 2% SRBC was added to each tube. The tubes were incubated for 3 hr at 37°C with periodic shaking to re-suspend sedimented organisms and erythrocytes. The 50% haemolysis end-point was read visually.

Estimation of haemolysis after removal of viable organisms (after Ginsburg and Harris, 1965): Glass tubing, 1 cm in diameter, was drawn out at one end and was plugged with glass beads and glass wool. The total length of the tubing above the glass wool was approximately 10 cm.

DEAE cellulose (Whatman DEI, W. and R. Balston Ltd., England) was suspended in 500 ml of distilled water and mixed thoroughly. After settling, the supernatant pH was adjusted to 7.2 - 7.3 with 0.1N HCl. The slurry was again mixed and if necessary the pH was re-adjusted. The supernatant was then removed and a thick slurry was made in V-C buffer, pH 7.3. This DEAE cellulose slurry was then poured into the glass columns. It was allowed to settle and was then packed with a vacuum pressure of 4 - 5 lb/in^2 so that each column contained packed DEAE cellulose to a height of 5 cm. The columns were then washed with V-C buffer for 1 - 2 hr at $4^{\circ}C$. Each batch of DEAE cellulose was only used once.

E. coli cells were grown in CDM according to the methods described for β-haemolysin production (page 41). The cell pellet from 1 hr, stage C cultures, was diluted to approximately 1.5×10^7 cells/ml with V-C buffer or V-C buffer containing a 1:32 dilution of CDM. To 0.5 ml volumes of these suspensions was added 0.5 ml of 10% SRBC and the mixtures were incubated at $37^{\circ}C$. At intervals up to 1 hr the mixtures were passed rapidly through prepared DEAE cellulose columns (see Figure 2). Effluent volumes of 10 ml were collected and were divided into two fractions of 5 ml. To one fraction was added 0.001% (w/v) merthiolate in saline and both mixtures were re-incubated at $37^{\circ}C$ so that the total incubation time from beginning to end of the experiment was 3 hr. Viable counts were performed at the start of the experiment (sample time I), immediately after passage through the columns (sample time II) and at the end of the experiment (sample time III). Suspensions of erythrocytes in diluent or CDM-diluent served as negative controls. The columns were carefully standardised so that, after collecting 10 ml effluent, less than 1% of erythrocytes had failed to percolate through the column. A standard curve for haemoglobin release was prepared by lysis of control effluents with saponin (100% lysis) and dilution with distilled water. Samples (2 ml) were removed from the test fractions at the same time

Figure 2. Flow diagram of procedure for interaction of β-haemolysin with sheep erythrocytes



as viable counts were performed; the samples were centrifuged for 2 min at 500 g and the $E^{0.5\ cm}_{545\ nm}$ of supernatants was measured as described previously. Percent haemolysis was determined by interpolation of the graph.

3. Kinetic haemolytic assay.

Standardisation of erythrocyte suspensions and calibration of the spectrophotometer: Kinetic studies were performed with silica cells of 1 cm light path (Unicam BS3875-1A) at $E_{650\ nm}$ in a controlled temperature Unicam SP800 spectrophotometer, to which was attached a Unicam SP22 chart recorder (Pye-Unicam, Cambridge, England) set at a 5-times multiplication factor. A 0.7% SRBC suspension was made in V-C buffer, pH 7.3. The spectrophotometer and chart recorder were then calibrated on a linear scale with the erythrocyte suspension. To 1.4 ml of V-C buffer was added 0.1 ml of 0.7% SRBC. The contents of the cell were mixed by inversion and read at $E^{1\ cm}_{650\ nm}$. The zero controls of the spectrophotometer and chart recorder were adjusted if necessary such that the $E^{1\ cm}_{650\ nm}$ was 0.40. This represented a 100% erythrocyte concentration. The same procedure was repeated by addition of 0.05 ml of 0.7% SRBC to 1.45 ml of V-C buffer. An $E^{1\ cm}_{650\ nm}$ of 0.20 represented a 50% erythrocyte concentration and the spectrophotometer, chart recorder and SRBC suspension were considered to be standardised. V-C buffer was used in the reference cell.

Assay procedure: In the test system 0.1 ml of haemolysin or dilution thereof was added to 1.3 ml of V-C buffer in a spectrophotometer cell and the solution was rapidly mixed by inversion of the cell. After preincubation for 1 min at 37°C, 0.1 ml of standardised 0.7% SRBC was added, the test cell was again mixed by inversion and was replaced in the spectrophotometer. Lysis of erythrocytes was measured by monitoring reduction in $E^{1\ cm}_{650\ nm}$. Rates of haemolysis were determined by measuring the slope over the linear portion

of haemolytic reaction curves. The preincubation time was shortened or lengthened merely by addition of erythrocytes when required. Alternatively, the incubation temperature was altered by adjustment of the thermostatically controlled water bath attached to the spectrophotometer; the effect of pH was studied using diluent and erythrocyte suspensions in sodium cacodylate - HCl (pH 5.0 - 7.4) or Tris (hydroxymethyl) aminomethane-HCl (pH 7.2 - 9.0) buffers to which was added 10 mM calcium chloride (see Appendix II). The EDTA (ethylenediaminetetraacetic acid) used in some experiments was an analytical grade reagent (B.D.H., Poole, England). It was made up to a final concentration of 5 mM in V-C buffer, pH 7.3.

D. Concentration of α-Haemolysin.

1. Ammonium sulphate precipitation at 50% saturation.

To crude culture filtrate, stirred gently in a flask at 4°C, was added small amounts of solid ammonium sulphate until 50% saturation was achieved using the nomogram described by Dixon (1953). The contents of the flask were stirred for 18 - 20 hr at 4°C when the fluid, which contained a precipitate, was centrifuged at 5,000 g for 20 min at 4°C. The supernatant was discarded and the precipitate was dissolved in a minimal volume of distilled water. Thereafter, the concentrated haemolysin was dialysed for 6 hr at 4°C in Visking dialysis tubing (Scientific Instruments Centre Ltd., London) against 2 or 3 changes of 2 litres of distilled water adjusted to pH 8.0 with 0.5N NaOH. This preparation was stored at -20°C until required and is hereinafter referred to as Stage I haemolysin.

2. Precipitation at acid pH with 0.005M acetate buffer.

Stage I haemolysin was dialysed at 4°C against 3 changes of 0.005M acetate buffer, pH 4.6 (see Appendix II), until a greyish brown precipitate had completely formed. The contents of the dialysis sac were centrifuged

at 5,000 g for 20 min at 4°C; the supernatant fluid was discarded and the precipitate was dissolved in a minimal volume of 0.01M Tris buffer which contained 0.1M NaCl. This preparation was stored at -20°C and is referred to as Stage II haemolysin.

3. Ultrafiltration.

Crude culture filtrate, stage I or stage II haemolysin, kept at 4°C, was passed under positive nitrogen pressure of 10 - 20 lb/in^2 through "Diaflo" ultrafiltration membranes (Amicon Corp., Lexington, Mass.). The filters are biologically inert anisotropic synthetic polymers. Each type is manufactured such that macromolecules of a given molecular size are either retained by or pass through the membrane. For example, "Diaflo" XM100A membranes, in general, exclude substances with a molecular weight greater than 1 x 10^5. Fig. 3 shows a diagram of the apparatus and a schematic cross-section of a typical ultrafiltration membrane. Membranes were selected so that α-haemolytic activity was retained and concentrated 5 - 10 times in the ultrafiltration cell.

This technique was also used in attempts to determine an approximate molecular size for the haemolysin. In addition, samples of nutrient broth medium were passed through "Diaflo" membranes in order to obtain information regarding the molecular size of medium components required for α-haemolysin production. In these experiments, components retained by the membrane were adjusted to the same volume and protein concentration (page 61) as the filtrate. Both fractions were sterilised by autoclaving at 121°C for 15 min; 0.2% (w/v) glucose was added and α-haemolysin production was measured as described (page 40).

E. Purification of α-Haemolysin.

1. Sephadex gel chromatography.

Figure 3 : Schematic diagram of Amicon ultrafiltration cell

Legend:

A. Ultrafiltration cell.

B. Stirring assembly.

C. 'Diaflo' membrane.

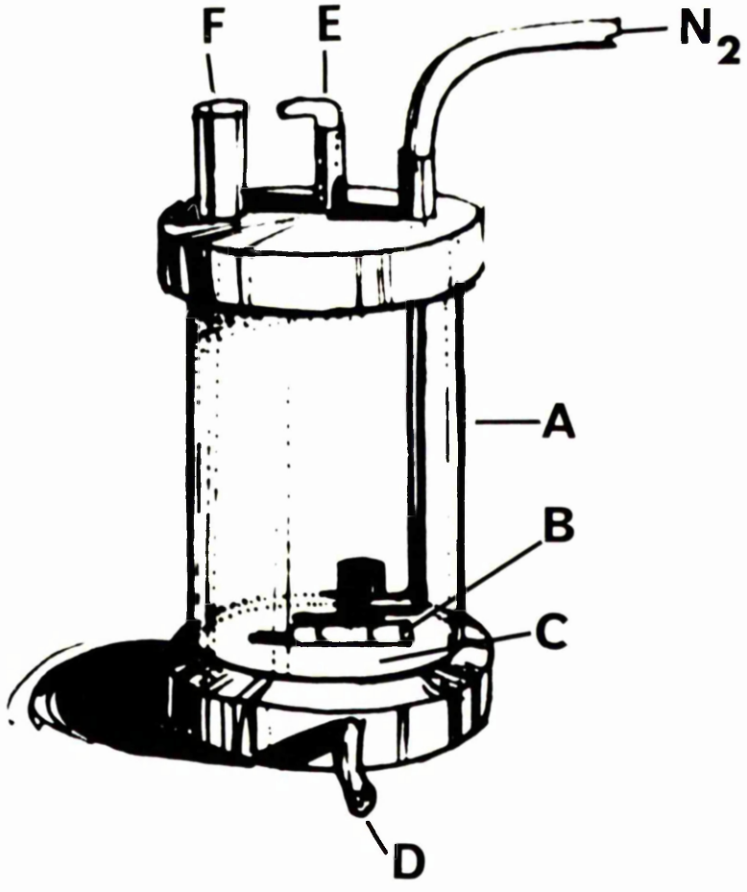
D. Outlet port.

E. Pressure relief valve.

F. Sample-inlet port.

G. Expanded view of a 'Diaflo' membrane showing that microsolutes (◉) pass through the membrane but macrosolutes (O) are rejected at the membrane skin (H) and remain in the cell.

N_2 - Nitrogen pressure.


F
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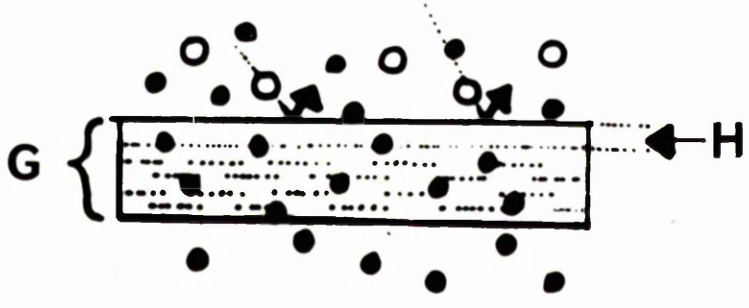

G
H

Principles: Although the manufacturers of Sephadex (Pharmacia Ltd., Uppsala, Sweden) provide detailed information on the theory and practice of Sephadex gel filtration, a brief summary of the principle will be presented here.

"Sephadex" is the registered trade mark of a series of beaded gels prepared by cross-linking selected dextran fractions from Leuconostoc mesenteroides with epichlorohydrin. Sephadex is strongly hydrophilic and swells easily in water and electrolyte solutions. The degree of cross-linking imparts differences in the swelling properties of Sephadex and thus substances can be fractionated according to molecular weight. In a column filled with swollen Sephadex of a particular grade, molecules which are larger than the largest pores of the gel beads (the exclusion limit) do not penetrate the gel and are eluted first. Smaller molecules, depending on size and shape, enter the pores to varying degrees and are eluted according to decreasing molecular weight. Each type of Sephadex fractionates within a particular molecular weight range, determined by the amount of swelling of the gel. Thus, the choice of Sephadex type depends on the molecular size of the substances to be separated.

Apparatus used: For all applications of Sephadex gel filtration in this investigation a Pharmacia K26/100 column (2.6 cm internal diameter; 100 cm long), fitted with top and bottom flow adaptors and a Mariotte flask, was employed. The flow adaptors provided by Pharmacia are constructed so that the sample was spread evenly over the surface of the Sephadex bed and furthermore, fractions were similarly eluted at the column outlet. Use of a Mariotte flask kept the operating pressure and flow rate constant during experiments. A schematic diagram showing the operational apparatus used and a cross-section of the sample applicator part of a flow adaptor is given in Figure. 4.

Figure 4 : Schematic diagram of Sephadex G-200 apparatus

Legend:

A. Mariotte flask reservoir.

B. Sample holder.

C. Three-way tap.

D. Constant operating pressure-head.

E. Flow adaptors, top and bottom.

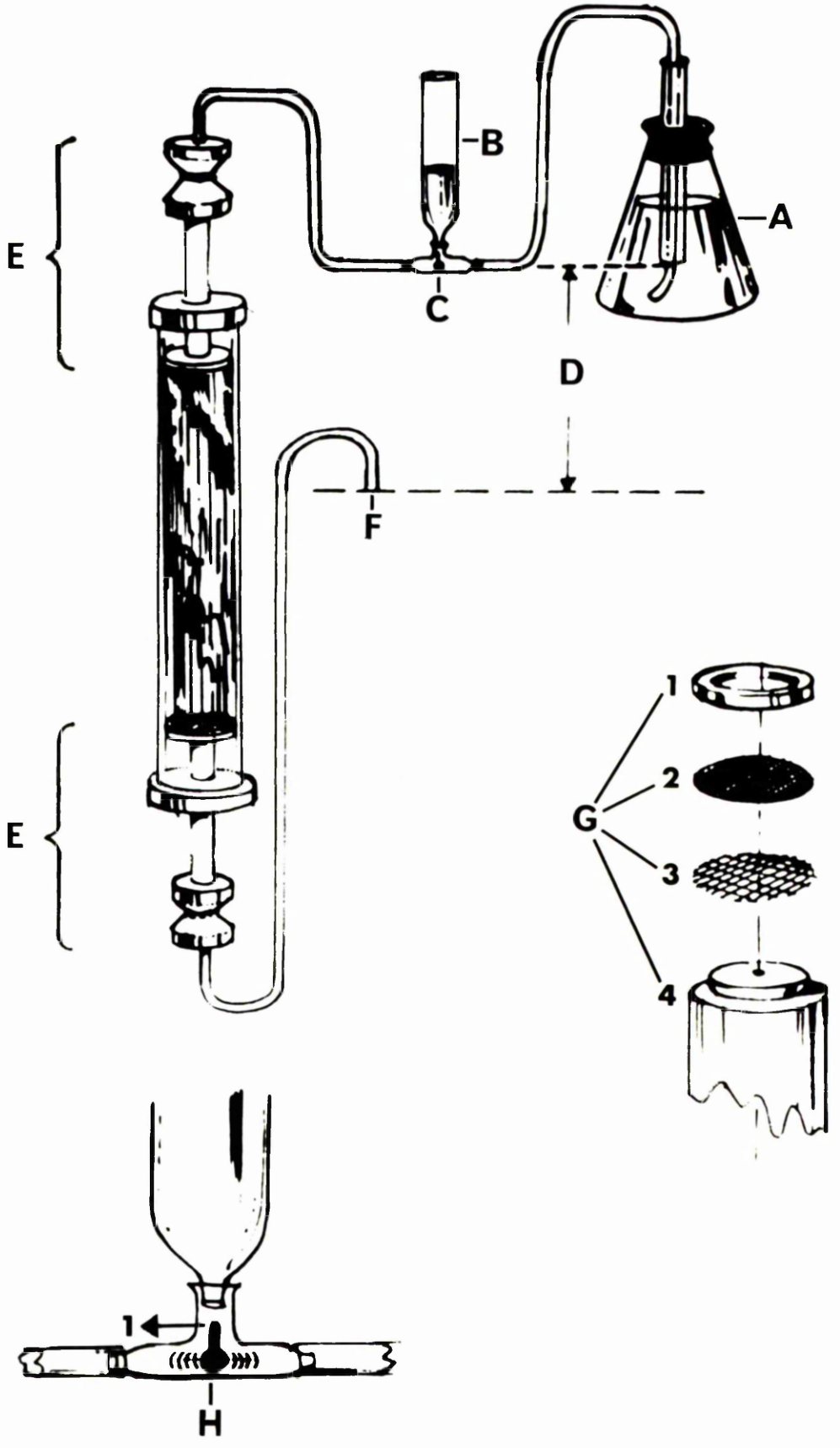
F. Column outlet.

G. Cross-section of sample applicator portion of a flow adaptor

1) Net fastener
2) Nylon net
3) Support screen
4) Flow adaptor plunger.

H. Expanded view of three-way tap

1) Movement of tap in the direction of the column allows application of sample.


A
B
C
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Preparation, packing and equilibration of the column: To 1 litre of distilled water was added 15 - 16 g of dry, medium grade Sephadex G-200. The amount added was determined by reference to the water regain value/g of Sephadex G-200 (30 ml/g) and the total effective volume of the column (480 ml) with the bottom flow adaptor in place (Figure 4). The Sephadex was allowed to swell at 80°C for 5 hr with occasional gentle stirring of the mixture. All further procedures were performed at 4°C. The swollen Sephadex was allowed to settle, the supernatant was decanted off and the gel was washed 3 times in cold eluant buffer (0.01M Tris buffer, pH 7.3) in order to remove fine particles of G-200 which did not sediment. A dilute slurry of the gel was then made in eluant buffer. The column, mounted vertically in the cold room, was partially filled with buffer and the slurry was poured into the column. The column outlet was opened and the gel was allowed to pack by gravity. When the column was filled with Sephadex the top flow adaptor was placed into position and the column was washed with 2 litres of 0.01M Tris buffer in order to complete packing of the gel and equilibrate the system. The top flow adaptor was then adjusted so that it rested directly on top of the gel bed with no air bubbles between the nylon net and the gel. The column was then ready for use.

Determination of the void volume (Vo): This measurement was performed for two reasons; first, as a check on how evenly the gel had packed in the column, and second, to determine the upper limit of the fractionation range of G-200 in this column. For this purpose 10 ml of a 0.5% (w/v) solution of Blue Dextran 2000 (Pharmacia Fine Chemicals), a dye-conjugated dextran which has an average molecular weight greater than 1×10^6, was applied to the column. The progress of the dyed compound was followed through the column to check for "tailing". Eluate was collected as soon as the three-way tap was returned to the reservoir-column flow position (Figure 4). When the Blue Dextran neared the bottom of the gel bed, 5 ml fractions were collected in an

LKB 7000 Ultrorac fraction collector (LKB Produkter, Bromma 1, Sweden). The $E^{1\ cm}_{625\ nm}$ of the fractions was measured in an SP600 spectrophotometer with glass cells. Eluant buffer was used in the reference cell. V_o was defined as the volume of fluid required for a substance, which is completely excluded by Sephadex G-200, to pass through the column. The $E^{1\ cm}_{625\ nm}$ peak of Blue Dextran 2000 was eluted in 145 ml of eluate. The height of the gel bed after complete equilibration was 83 cm.

Eluant buffers: Two modified eluant buffers were used for gel filtration studies. All contained 0.01M Tris buffer, pH 7.3 with 0.001% merthiolate (w/v) as a preservative. To the Tris eluant was added sodium chloride to 0.1M final concentration (TS buffer) or 0.1M sodium chloride and 5% (v/v) glycerol (TSG buffer). When the eluant was changed, 2 litres of modified buffer were washed through the column to re-equilibrate it.

Sample application, elution and monitoring of fractions: Stage II haemolysin was purified by Sephadex G-200 chromatography. Ten ml of sample was pipetted into the sample holder (Figure 4). The tap was turned in the direction of the column and the column outlet was opened. When the sample holder was almost empty the tap was returned to the vertical position and bulk eluate was collected in a measuring cylinder. For all experiments the operating pressure of the system was kept at 20 - 25 cm and the flow rate remained at 15 - 18 ml/hr (i.e. approximately 3 $ml/cm^2/hr$). After collecting 100 ml of eluate, the outlet tubing was attached, at the same operating pressure height, to the LKB fraction collector and 5 ml fractions were collected.

Fractions were monitored for protein content at $E^{1\ cm}_{280\ nm}$ in a Unicam SP500 spectrophotometer with silica cells. Eluant buffer was employed in the reference cell. Haemolytic titrations were performed as described previously.

2. Iso-electric focusing.

The theoretical and practical considerations of the technique of iso-electric focusing have been reviewed in detail by Smyth (1972). It is only necessary here to describe the experimental procedures used in this thesis.

In all experiments the 110 ml (LKB 8101) column was used with the appropriate pH gradient mixer (LKB 8121) and peristaltic pump (LKB, 10200). The column and its accessories were set up as described by Smyth (1972) and McNiven (1972).

Preparation of samples for electrofocusing: The presence of high concentrations of salt will disturb the pH gradient in the column. Therefore, Stage I haemolysin, which was used for electrofocusing studies, was dialysed overnight at 4°C with stirring against several changes of 30 volumes of 1% (w/v) glycine (BDH-Analar) in distilled water, pH 6.15. Alternatively the sample was precipitated by dialysis against distilled water adjusted to pH 4.0 with HCl and dissolved in 3.5M urea for application to columns containing urea.

The electrofocusing procedure: The preparation of sucrose gradients in the presence and absence of urea is described in Appendix III. The volumes of dense and light solutions used were those of Bernheimer, Grushoff and Avigad (1968) which ensured that all the sample was pumped into the column. Sample solutions were added to the light gradient solution. All solutions were brought to 4°C before preparation of the gradient in the column.

Concentrated carrier ampholytes were used to prepare pH gradients between pH 3 - 10 (LKB 8141) and pH 3 - 6 (LKB 3152). After a 4-fold dilution of the 40% (w/v) concentrates in distilled water, diluted ampholine was added to the dense and light solutions (see Appendix III).

Preparation of electrode solutions, loading of the column and electrofocusing run conditions were similar to those described by McIlven, Owen and Arbuthnott (1972). In this study, the sample was always added to the light solution.

Fractions of 2 ml were collected when electrofocusing was complete; pH measurements were made using a Vibret pH meter (E.I.L., Surrey, England). Smyth (1972) has discussed in detail the effect of urea on the pH of solutions and as recommended by this author the pH of fractions containing urea was determined using standard buffer dissolved in 3.5M urea. The $E^{1\ cm}_{280\ nm}$ of all fractions was measured in a Unicam SP500 spectrophotometer with silica cells. Distilled water was employed in the reference cell.

F. Immunological Methods.

1. Preparation of antiserum.

Antiserum to α-haemolysin was produced in rabbits according to the schedules of Smith (1963) and Gallop et al., (1966). For the former method stage I haemolysin was used as the antigen; in the latter method stage II haemolysin was employed. The schedules used for antiserum production are given in Table 10. Antiserum obtained was stored at -20°C until required, when it was inactivated by heating at 56°C for 30 min and filtered as a sterility precaution.

2. Haemolysin neutralisation on agar plates.

Qualitative estimations of the ability of antiserum preparations to neutralise zones of haemolysis on agar plates were performed by a modification of the method of Elek and Levy (1950). In the overlay part of sheep erythrocyte overlay medium (Appendix I), wells (1 cm x 4 cm) were cut with a sterile scalpel. The agar in the wells was removed and the space

Table 10 : Schedules for production of antiserum to α-haemolysin

Schedule	Day	Route	Antigen Preparation
I (after Smith, 1963)	1, 2 and 3	intravenous	1.0 ml stage I haemolysin containing 0.1, 0.5 and 1 x 10^3 IU.
	7, 8 and 9	intravenous	1.0 ml stage I haemolysin containing 2.5 x 10^3 IU.
	14	-	None: Test bleed
	15	-	None: Bleed out
II (after Gallop et al., 1966)	1, 3, 5 and 10	subcutaneous	0.2 mg stage II haemolysin in Complete Fruend's Adjuvant
	19	-	None: Test bleed
	41, 43 and 45	intravenous	0.3 mg stage II haemolysin
	50	-	None: Test bleed
	51	-	None: Bleed out

between the erythrocyte overlay and the nutrient agar base was sealed with a small amount of sterile nutrient agar. The wells were filled with antiserum and the petri dishes were left overnight at room temperature on a flat surface in order to allow diffusion of antiserum into the medium. The following day test strains were streaked at right angles to the wells. After overnight incubation at 37°C, inhibition of zones of haemolysis was recorded in mm. The original method of Elek and Levy using filter paper strips soaked in antiserum and sunk into blood agar plates was found unsatisfactory due to extensive growth of motile E. coli strains around the filter paper strips.

3. Haemolysin neutralisation by tube test.

More precise determinations of the potency of anti-α-haemolysin were made using a neutralisation test in tubes. First, the activity of the α-haemolysin sample was determined (page 43). Second, serial doubling dilutions of heat-inactivated antiserum were made in 0.5 ml volumes of V-C buffer. To each tube was added 0.2 ml of diluted α-haemolysin (i.e. 20 HU/ 0.2 ml). Pre-immune serum was treated in a similar manner. After 1 hr at 4°C, 0.3 ml of a standardised 3% SRBC suspension was added to each tube and these were incubated for 1 hr at 37°C. The last tube showing complete neutralisation was taken as the end point; the number of anti-haemolysin units was recorded as the reciprocal of the antiserum titre multiplied by the number of haemolytic units used in the test.

To test for the neutralisation of β-haemolysin, haemolytic cells were prepared as described (page 41) and were diluted in V-C buffer to contain between 1×10^5 and 1×10^7 viable cells/ml. To these suspensions was added 0.2% (w/v) α-D-glucose. Cells thus prepared were then treated with anti-α-haemolysin antiserum in the same way as described for neutralisation of α-haemolysin, except that tubes were incubated for 3 hr at 37°C.

4. Immunodiffusion.

Ouchterlony-type immunodiffusion was performed according to the method of Crowle (1958) on microscope slides fitted with perspex cover slips
Ouchterlony-type immunodiffusion was performed according to the
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method of Crowle (1958) on microscope slides fitted with perspex cover sli
each end so that there was a space between the cover slip and the slide. Ionagar (Oxoid), 0.75% (w/v) in barbitone buffer, pH 8.3 (see Appendix I), was applied under the cover slips, and the prepared immunodiffusion slides were left in a moist chamber for 30 min at room temperature. Antiserum to stage II haemolysin was injected, using a fine needle, into the centre well of the cover slip and haemolysin samples were applied to surrounding wells. Extreme care was required to ensure that no air bubbles remained in the wells between the cover slip and the agar on the slide. After 24 hr at room temperature in the moist chamber the cover slips were removed and the slides were washed gently overnight in 0.01M phosphate buffer pH 7.5 (see Appendix II) in order to remove soluble material from the agar. The agar was then stained for 15 min with saturated nigrosine in 2% (v/v) acetic acid. Destaining was performed with an aqueous solution of 2% acetic acid in 1% glycerol (v/v).

Diffusion coefficient: If an antigen and antibody are applied at 90^{o} to one another in troughs cut in agar, the precipitin line which forms can be used to estimate the molecular weight of the antigen by determination of the diffusion coefficient (Allison and Humphrey, 1960). Diffusion coefficients (D_{20}) of antigens with known molecular weights are used for comparison. The formula for calculation of the diffusion coefficient is :

$$\left(\frac{D_{AG}}{D_{AS}}\right)^{\frac{1}{2}} = \tan \theta$$

when θ is the angle between the precipitin line and the antigen trough; D_{AS} is taken as $3.8 \times 10^{-7} cm^{2} sec^{-1}$ (human γ-globulin).

Haemolysin purified by Sephadex gel filtration was precipitated by dialysis against 85% saturated ammonium sulphate. The precipitate was resuspended in 0.01M Tris, 0.1M potassium chloride buffer, pH 7.2 to one-fifth the original volume. Ionagar at 1% (w/v) concentration was prepared in this buffer and 0.001% merthiolate was added. The agar was melted and 15 ml was pipetted onto a glass slide (4 x 5 cm) onto which was mounted 2 glass microscope slides set at right angles with a space of 3 mm between them. When the agar had set the microscope slides were removed. The concentrated haemolysin preparation was applied to one of the wells left by the imprint of the microscope slide; heat-inactivated antiserum was applied to the other well. The plate was left in a moist chamber until a precipitin line formed. The angle to 90^{o} of this line was measured and the diffusion coefficient was calculated from the mean of duplicate estimations.

G. Electrophoretic Methods.

1. SDS disc-gel electrophoresis: SDS (sodium dodecyl sulphate) disc-gel electrophoresis was performed by a modification of the method of Neville (1967). The separating gel contained 3.5% acrylamide, 0.2% NN'-methylene-bisacrylamide, 9M urea and 2% SDS at pH 4.9. No stacking gel was used.

Preparation of the tank buffers, gel buffers and catalysts are given in Appendix IV. Samples were prepared by the addition of 0.1 ml of an aqueous solution of 8.3% (w/v) glycine and 12M urea to 0.1 ml of haemolysin. After 30 min at room temperature, 0.04 ml of an aqueous 20% (w/v) solution of SDS and 8 drops (0.15 ml) of glycerol were added. Aliquots (0.15 ml) of the prepared samples were injected with a 26 gauge, 3/8 in needle attached to a 1 ml syringe onto the surface of the gel columns. A current of 1.5 mAmp per gel was applied. It was found initially that, under the conditions used, the tracking dye remained in the upper tank buffer.

Therefore, cytochrome C (see Appendix IV), applied to separate gel columns at a concentration of 15 µg/ml, was employed in place of the dye marker. When the cytochrome C had migrated to within 0.5 cm of the bottom of the gel the current was switched off. Gels were removed from the columns using a syringe filled with distilled water. They were fixed and stained by overnight immersion in a solution of amido black dissolved in acetic acid/ methanol (Appendix IV). Gels were rehydrated in 7% (v/v) acetic acid and electrophoretically destained in 7% acetic acid at 6 mAmp per gel. Standard proteins (see Appendix IV) were run under these conditions in attempts to compare the molecular weight of urea/SDS-treated haemolysin with identically treated proteins of known molecular weight.

H. Chemical Assay Procedures.

1. Protein estimation.

Protein estimations were performed by the method of Lowry et al., (1951) using bovine serum albumin (BSA, fraction V; Armour Pharmaceuticals, England) as a standard. In addition, protein content was monitored by ultraviolet absorption measurements at $E^{1\ cm}_{280\ nm}$ using silica cells in an SP500 spectrophotometer. Appropriate buffers or distilled water were employed in the reference cell.

2. Quantitative determination of carbohydrates.

Estimation of reducing sugars: Estimations of reducing sugars were determined according to the method of Nelson (1944). The reagents were prepared as described in Appendix V. For quantitative determinations of reducing sugars 25 parts of reagent A were added to 1 part of reagent B. A standard curve was prepared using α-D-glucose (BDH-Analar), 20 - 200 µg. In the test procedure, 0.05 ml of samples were made up to 1.0 ml with distilled water in 6 x 5/8 in chromic acid cleaned test tubes. One (1.0) ml of Nelson's

reagent was added and the tubes were incubated in a boiling water bath for 20 min. After cooling to room temperature, 1.0 ml of arsenomolybdate reagent and 15 ml of distilled water were added. The samples were monitored at $E^{1\ cm}_{650\ nm}$ in the SP600 spectrophotometer with glass cells. The amount of reducing sugar was determined by interpolation of the graph. Samples which contained only distilled water served as controls.

Total hexose sugars: Total hexose was determined colorimetrically by the anthrone method (Scott and Melvin, 1961). Anthrone reagent was prepared as described in Appendix V. 'Analar' α-D-glucose, 10 - 100 µg, was used to prepare a standard curve. Test samples (0.05 ml) and dilutions of glucose were made up to 3.0 ml with distilled water. Distilled water with no additives, served as the reagent blank. The samples, in 6 x 5/8 in chromic acid cleaned test tubes were placed in an ice bath and 6 ml of acidified anthrone reagent was added to each tube. Extreme care was taken so that the mixture did not spurt out of the tubes. The tubes were transferred to a boiling water bath for 3 min. After cooling to room temperature, samples were read at $E^{1\ cm}_{625\ nm}$ in the SP600 spectrophotometer with glass cells. As with the Nelson method, the amount of hexose was determined by interpolation of the graph.

3. Analysis for phosphate by a modified Allen method (1940).

This method depends on the conversion of all phosphorus to inorganic orthophosphate by digestion with sulphuric acid and hydrogen peroxide. The reduction of a phosphomolybdate complex, formed by reaction with ammonium molybdate, with amidol (2;4-diaminophenol hydrochloride) gives a characteristic blue chromogen. The intensity of this colour is measured spectrophotometrically at 640 nm. The "½ Allen" method used in this study allows quantitative determination of 0 - 150 µg phosphate.

Standard phosphate solutions and other reagents are given in Appendix V. Samples for assay were pipetted into microkjeldahl flasks which contained 2 or 3 glass beads. To each flask was added 0.6 ml of 10N H_2SO_4; the necks of the flasks were washed with distilled water and gentle heat was applied until the glass beads began to vibrate. At this point the heat was turned up and digestion was continued until the contents of the flasks began to fume. When organic material was present the contents of the flasks blackened. When cool, a small amount of H_2O_2 was added and heat was re-applied. This procedure was repeated until the contents were clear.

After cooling to room temperature, 10.4 ml of distilled water, 1.0 ml of amidol reagent and 0.5 ml of 8.3% ammonium molybdate were added. The flasks were allowed to stand for 10 min but no longer than 30 min when the $E^{1\ cm}_{640\ nm}$ was measured using glass cells. Distilled water was used in the reference cell. The amount of phosphorus, as inorganic orthophosphate, in each sample was determined by comparison with a standard curve prepared with 0 - 150 µg quantities of a standard inorganic phosphate solution.

4. Estimation of DNA (deoxyribonucleic acid).

Determination of DNA in haemolysin preparations was performed by the diphenylamine method of Burton (1956). The method depends on dehydration and rearrangement of 2-deoxyaldopentoses to yield ω-hydroxylevulinic aldehyde. In DNA only purine nucleotides react; pyrimidine nucleotides are not split in the reaction. The product of the reaction between ω-hydroxy-levulinic aldehyde and diphenylamine is a blue chromogen which is measured spectrophotometrically at $E_{600\ nm}$.

The preparation of reagents for this estimation is given in Appendix V. To 1 ml of solution in 6 x 5/8 in tubes was added 2 ml of the diphenylamine reagent. The tubes were incubated at 30°C for 20 hr. Estimation of DNA content after measurement at $E^{1\ cm}_{600\ nm}$ in glass cells was made

by interpolation of a standard curve prepared by addition of 0 - 100 µg of commercial DNA (ex-calf thymus, Miles-Seravac, England) to the diphenylamine reagent.

I. Biological Assays.

1. Analysis for phospholipase activity in α-haemolysin preparations. The ability of α-haemolysin to hydrolyse lecithin and sphingomyelin was tested. L-α-lecithin (General Biochemicals, Lot No. 84781) was supplied by Smyth (1972); sphingomyelin was obtained from Sigma, London. Emulsions of the substrates were made in V-C buffer containing 1 mg/ml BSA (bovine serum albumin, Fraction V; Armour Pharmaceuticals, England). Dispersion of the emulsions was aided by sonication in an ultrasonic cleaner (Allan and Hanbury - Model 150 - Surgical Instruments, London). Reaction mixtures contained 1 mg of lecithin (42.6 µg phosphorus) or 1 mg of sphingomyelin (38.6 µg phosphorus) (Dawson et al., 1969) in a final volume of 1.0 ml. Duplicate reaction mixtures contained 0.5 ml of emulsion, 0.4 ml of V-C buffer and 0.1 ml of α-haemolysin. Distilled water was substituted for haemolysin in the controls. Tubes were incubated at 37°C for 24 hr. The contents of one tube were analysed for water-soluble phosphorus by the "½ Allen" method (page 62). To the other tube 10 ml of chloroform (Analar) was added. After separation of the phases, the extracts were evaporated to dryness and redissolved in 0.2 ml chloroform. Thin layer chromatography (TLC) of 5 µl volumes of test and control extracts was performed using Silica Gel G (Merck). Plates were developed in chromatography tanks (Shandon, London) with chloroform: methanol:water (130:70:10). After drying, the plates were sprayed with 50% (v/v) sulphuric acid and heated at 110°C for 10 min. Phospholipids were detected as charred spots.

2. The effect of α-haemolysin in tissue culture.

Tissue culture cells: HeLa and BHK (baby hamster kidney) cells were kindly

provided by Dr. T. H. Birkbeck of this department as monolayers in screw capped test tubes. The Eagles medium (Biocult Laboratories, Scotland) supplemented with calf serum (Biocult) on which they were maintained was pipetted off aseptically.

Test procedure: Dilutions of stage II α-haemolysin, which contained 10^2 - 10^5 HU/ml, were prepared in TS buffer (page 54). To 0.5 ml volumes of diluted haemolysin were added 0.5 ml volumes of Eagles maintainance medium which contained 200 units of cristamycin (Glaxo Laboratories, England) and 4 mM glutamine (Biocult). Calf serum was omitted. The mixtures of Eagles medium and haemolysin (1.0 ml) were then pipetted into the tubes containing the monolayers of cells. After overnight incubation at 37°C, the neutral red assay (see below) was performed. In tests designed to determine the effect of calcium ions on tissue culture toxicity, calcium chloride was added to the system such that final concentrations of 0 - 20 mM calcium chloride were included. Control cells contained only Eagles medium diluted with an equal volume of TS buffer containing the desired concentration of calcium chloride.

Neutral red assay for cell death: This assay is based on the amount of neutral red dye taken up by cells in a monolayer. Live cells take up the dye; dead cells do not (Finter, 1969; Greaves, Potter & McEntegart, 1971).

After overnight incubation at 37°C with α-haemolysin the medium was removed and 1.0 ml of a 0.003% solution of neutral red (Gurr, London) in Dulbecco's A saline (Appendix II) was added to each tube. After a further 2 hr at 37°C the neutral red was removed and the cells were washed with Dulbecco's A saline to remove excess dye. The cells were then treated with 1.0 ml of a 50% (v/v) ethanol-sodium citrate solution, pH 4.0 to extract the dye from viable cells. $E^{1\ cm}_{540\ nm}$ measurements of such extracts were made in the Unicam SP500 with glass cells.

3. Lethality titrations in mice.

The lethal activity of α-haemolysin was tested in adult Porton White mice (Tuck and Son Ltd., Essex, England) weighing 18 - 20 g. Stage II haemolysin was diluted in V-C buffer to contain 0.035 - 3.5 mg/ml protein as determined by Lowry estimation (page 61). A volume of 0.1 ml of each concentration was injected intravenously with a 26 gauge, 5/8 in needle attached to a 1 ml tuberculin syringe into the tail veins of groups of 4 mice. Control mice received either V-C buffer alone or the highest concentration of haemolysin which was inactivated by heat at 56°C for 1 hr. The number of survivors was recorded at 24 and 48 hr.

4. Intradermal injection in rabbits.

The method of Moon and Whip (1971), designed to test E. coli enterotoxins for vascular permeability factors was used to examine the effect of α-haemolysin after intradermal injection into the skin of rabbits.

The backs of young New Zealand White Rabbits of either sex (Hyline Commercial Rabbits, Cheshire, England) weighing 2.0 - 2.5 kg, were shaved with clippers. Stage II haemolysin was diluted as described for lethality titrations in mice (see above). Sephadex-purified α-haemolysin was used as an undiluted solution. The shaved backs of 4 rabbits were marked in a square grid with a wax marking pencil. Injections of 0.1 ml of haemolysin preparations were made intradermally with a 26 gauge, 5/8 in needle attached to a 1 ml tuberculin syringe. Each dose of haemolysin was injected at different sites into the backs of the rabbits. Sterile control preparations which were injected included saline, V-C buffer, TSG eluant buffer (page 54), heated stage II haemolysin (350 μg) and sterile nutrient broth concentrated by the methods used for the preparation of stage II haemolysin.

After 18 hr the rabbits were injected intravenously with volumes of a sterile 2% (w/v) saline solution of Evans Blue dye (Gurr, London) equal to 40 mg/kg. One hr later the rabbits were examined for areas of swelling and increased intensity of blue colour around the injection site. Zones of swelling and/or blueing were recorded in mm as the average of four separate measurements.

5. Ligated intestinal loop test in rabbits.

Ligated intestinal loop tests in experimental animals have been used to examine the enterotoxic potency of enteropathogenic E. coli and enterotoxin preparations from these organisms (see Introduction; section B:3). The method depends on the capacity of the organism or isolated enterotoxin to cause an accumulation of mucous fluid in ligated loops of small intestine; the potency of preparations is determined by the volume of fluid which is extracted from loops at autopsy.

Enterotoxic potency of the following preparations was examined in ligated intestinal loops prepared in rabbits after the method of Smith and Halls (1967b) :

1. Stage II α-haemolysin.
2. Strain 25238, the haemolysin-producing E. coli strain.
3. E. coli, serotype 055:K59(B5):H6 (NCTC 8603, Aberdeen β), a non-haemolytic, enteropathogenic strain tested by Taylor et al., (1961).

The test strains were grown overnight at 37°C in NBG medium. Before injection they were diluted 1:10 in fresh NBG medium.

Surgical procedures were kindly performed by Mr. I. Duncan, Department of Veterinary Surgery, University of Glasgow. The abdominal area

of young New Zealand White Rabbits (Hyline Commercial Rabbits, Cheshire, England) weighing 2.0 - 2.5 kg, was shaved. The animals, starved for 24 hr before surgery, were anaesthetised with a mask of 3% Fluothane ^{R} (Imperial Chemical Industries Ltd., England) and nitrous oxide and were maintained under anaesthesia throughout the surgical procedure with 1% Fluothane and nitrous oxide. Strict aseptic precautions were observed during surgery. Sterile surgical draping was used and the abdominal area was swabbed thoroughly with antiseptic.

A midline incision, 6 - 7 cm long was made and the anterior portion of the small intestine located. Initial experiments confirmed the observation of Smith and Halls (1967b) that the first and last 40 cm of intestine were unsuitable. The useable portion, approximately 100 - 120 cm, was divided by means of string ligatures into test segments, 8 - 10 cm long, separated by shorter (4 - 5 cm) control segments. The test strains or stage II haemolysin were injected in 2 ml volumes into the lumen of the test segments. Haemolysin inactivated by heating at 56^{o}C for 1 hr and sterile saline were also included. Control segments were left uninjected. Care was taken not to damage any intestinal vasculature when injecting the loops. The abdominal wound was then closed in two stages with gut and nylon sutures and the animals were allowed to recover from the anaesthetic.

During the next 20 hr the rabbits were examined periodically for signs of agony in which case they were destroyed immediately. Animals which survived were killed by intracardiac injection of a fatal dose of sodium pentobarbitone. The abdomen was opened immediately and the small intestine was excised and unravelled. The macroscopic appearance of test and control segments was noted and the loops were examined for fluid exudate.

Samples from test and control loops, and from the heart blood,

were taken. These were streaked on to MacConkey's agar and nutrient agar medium. Colonies from nutrient agar plates were tested by presumptive serological analysis to confirm the identity of the isolated organisms.

RESULTS

RESULTS

A. Survey of the Haemolytic Activity of E. coli.

There were two reasons for surveying the haemolytic activity of E. coli isolates. First, it was advantageous to obtain a number of haemolytic strains in order to select a strain suitable for large scale haemolysin production; second, since reports in the literature of an association between certain serotypes of E. coli and human infections are usually not accompanied by descriptions of the haemolytic activity of these strains, it was desirable to investigate this aspect further. Accordingly, 181 E. coli isolates from inpatients and outpatients from both the Royal Hospital for Sick Children and the Queen Mother's Hospital, Yorkhill, Glasgow, were screened for haemolytic activity.

1. Bacteriological findings.

Preliminary studies showed that incorporation of washed sheep erythrocytes and calcium chloride into a single layer of nutrient agar medium gave, with some strains, indistinct zones of haemolysis which were difficult to score definitely as positive or negative. Therefore, an erythrocyte agar overlay medium (see Appendix I) was devised. With this medium strains could easily be designated as haemolytic or non-haemolytic.

The results of the survey of inpatients and outpatients from the above-mentioned hospitals are recorded in Table 11. The incidence of haemolytic activity among strains from inpatients was three times that of strains from outpatients.

An analysis of E. coli strains from inpatients in relation to the anatomical site of isolation (Table 12) revealed that 10 (71%) of 14 strains obtained from nose, mouth and throat swabs were haemolytic; infections of

Table 11 : Isolation of haemolytic *E. coli* from inpatients and outpatients.

Source of Isolate	No. of Isolates	No. Haemolytic	% Haemolytic
Inpatients	159	66	41.5
Outpatients	22	3	13.6
Totals	181	69	38.1

Table 12: Anatomical source of haemolytic *E. coli* isolated from inpatients.

Source of Specimen	No. of Isolates	No. Haemolytic	% Haemolytic
Nasal, mouth and throat swabs	14	10	71
Mid-stream urines	78	38	49
Wounds and abscesses	9	4	44
Genital tract (female)	7	2	28
Faeces and rectal swabs	47	11	25
Peritoneal swabs and gastric aspirates	4	0	0
Spinal fluids	2	0	0
Totals*	161	65	

* One blood culture yielded a haemolytic strain of *E. coli*.

the urinary tract, wound infections and abscesses also yielded a large number of haemolytic strains. By contrast, only 25% of isolates from faecal specimens were haemolytic. It is important to note that, of the 44 faecal strains, 37 were isolated from patients with confirmed gastroenteritis; 8 of these 37 strains were haemolytic. In addition, 2 of 9 enteropathogenic strains obtained from NCTC, Colindale, for reference, were haemolytic; these strains were not included in Table 12 because they did not form part of the survey of hospital strains.

It should be stressed that, except for the 37 faecal isolates from cases of gastroenteritis, information regarding the anatomical source of the strains of E. coli examined in this survey was not sought until haemolysis and agglutination tests had been performed. This protocol was observed in order to avoid bias.

2. Presumptive serological analysis.

The strains of E. coli isolated from inpatients were tested against antisera to known E. coli serotypes. It must be emphasised that, because of the high cost of agglutinating antisera, only slide agglutination tests using unheated cell suspensions could be done, allowing detection only of envelope antigens. Confirmation of O and H antigens would require a detailed serological analysis which was outwith the scope of this thesis.

The results of this part of the investigation are shown in Table 13. Of strains which agglutinated in OB antisera prepared against serotypes O86:B7 and O128:B12, 83% and 63% respectively were haemolytic. Also, 50% of strains which agglutinated in OB antisera prepared against serotypes O26:B6 and O55:B5 were haemolytic. Notably, all of the 11 isolates which agglutinated in antiserum prepared against serogroup O6 were haemolytic and 9 of these strains were isolates from urinary tract infections. By contrast, of 35 strains considered non-typeable by the methods used, only 6 (17%) were haemolytic.

Table 13 : Presumptive serological analysis of *E. coli* strains isolated from inpatients.

Antiserum to:	No. of Strains giving Agglutination		No. of Strains Haemolytic
	Haemolytic	Non-haemolytic	
Anteropathogenic serotypes:			
018:B21	1	0	1/1
020:B7	1	0	1/1
020:K84(B)	0	0	-
026:B6	3	3	3/6
028:B18	0	0	-
044:K74	0	1	0/1
055:B5	4	4	4/8
086:B7	10	2	10/12
0111:B4	0	0	-
0112:B11	0	0	-
0114:K	0	2	0/2
0119:B14	4	8	4/12
0124:B17	0	0	-
0125:B15	1	0	1/1
0126:B16	1	3	1/4
0127:B8	3	5	3/8
0128:B12	12	7	12/19
Other:			
06	11	0	11/11
019	1	0	1/1
Non-typeable[+]	6	29	6/35
Totals[x]	58	64	58/122

[+] Strains designated non-typeable did not agglutinate with any of the antisera which were available.

[x] Faecal isolates from diagnosed cases of gastroenteritis having known serotypes were not included in this analysis.

From the haemolytic strains of E. coli obtained from this survey, a single strain, 25238, serotyped as O19:B7, and of unknown flagellar type, was selected for further studies because it showed large zones of haemolysis on erythrocyte agar plates and consistent production of haemolytic activity when supernatant fluids or whole cells were titrated in a tube haemolysis test.

B. Studies on β-Haemolytic Activity.

1. Production of β-haemolysin.

It was considered initially that haemolytic activity observed in supernatant fluids of the test strain, when grown in Oxoid Tryptone Soya broth containing 0.2% (w/v) glucose, might be due to α-haemolysin. For this reason, investigations were continued using CDM (see Appendix I); this defined medium would offer obvious advantages in any subsequent purification procedures. However, when active supernatant fluids were filtered through 0.45 μ Millipore membranes, haemolytic activity was often lost completely.

In order to obtain high yields of haemolytic activity in whole cells and culture supernatants, and thus increase the possibility of finding measureable activity in culture filtrates, a three-stage culture method was devised (Rennie and Arbuthnott, 1971). From Figure 5 it can be seen that under normal growth conditions (stage A) haemolytic activity in supernatant fluids was low ($1 - 2 \times 10^2$ HU_{50}/ml); levels of cell-associated, β-haemolytic activity were only slightly higher. By removing these cells to fresh medium which contained no utilisable carbon source (stage B) haemolysin production ceased and no growth occurred. Addition of 0.2% (w/v) glucose, as carbon source, to these starved cells (stage C) resulted in immediate elaboration of haemolytic activity equivalent to maximum levels obtained in stage A cultures; activity then increased within 1 hr to 4 or 5 times the levels found in stage A

Figure 5 : Production of β-haemolysin and supernatant haemolytic activity by strain 25238 in CDM.

E. coli strain 25238, was grown in CDM by the three-stage method (see page 41). At the end of stage A (3.5 hr), cells were removed by centrifugation and re-suspended in fresh defined medium which did not contain glucose (stage B). After a further 3 hr at 37°C, 0.2% (w/v) glucose was added (stage C).

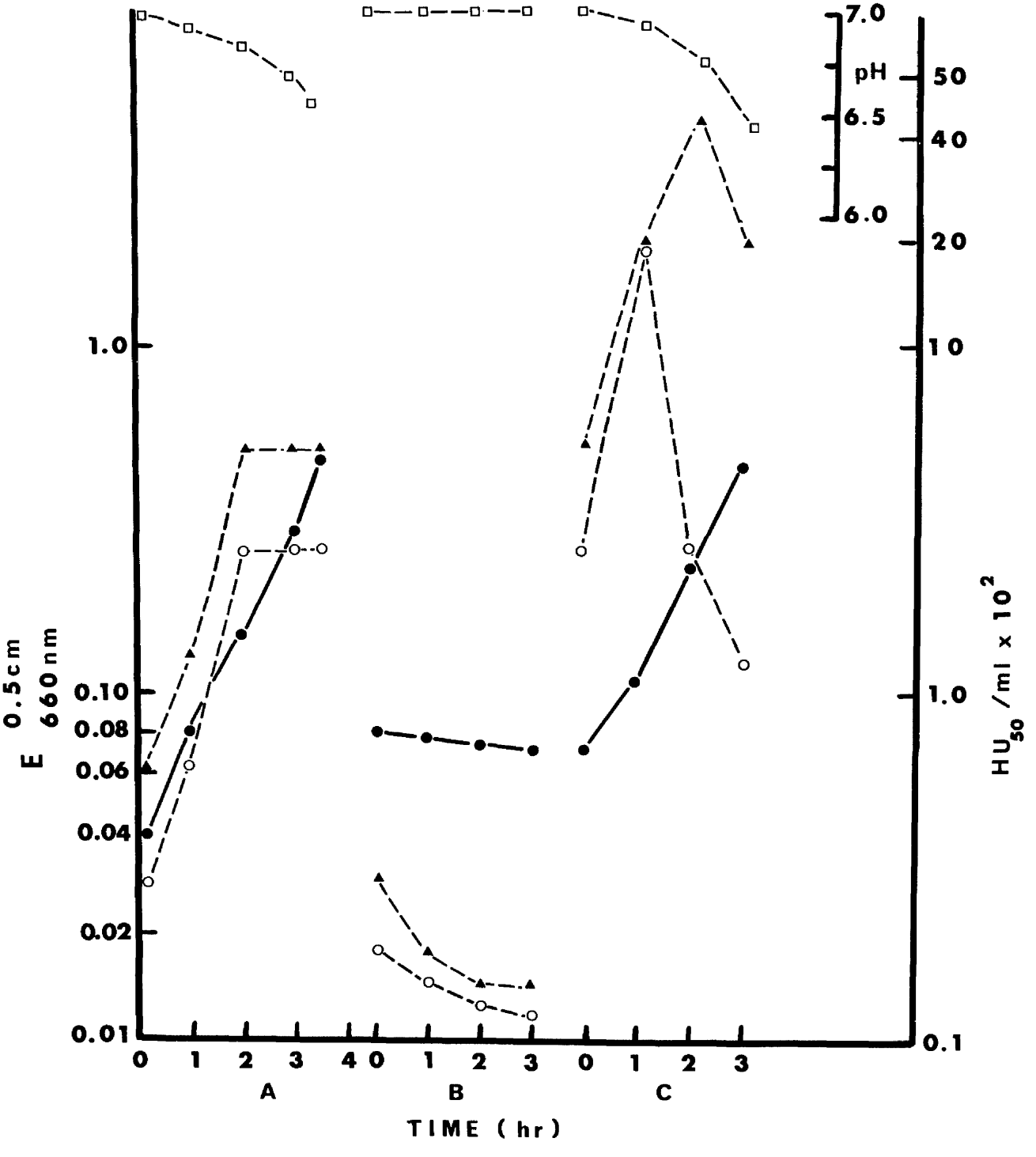
Symbols:

● : Growth as $E^{0.5\ cm}_{660\ nm}$

▲ : β-haemolysin (HU_{50}/ml x 10^2)

○ : Supernatant haemolytic activity (HU_{50}/ml x 10^2)

□ : pH


7.0
pH
6.5
6.0
50
40
20
10
1.0
0.1
E 0.5cm 660nm
1.0
0.10
0.08
0.06
0.04
0.02
0.01
HU_{50} /ml x 10^2
0 1 2 3 4 0 1 2 3 0 1 2 3
A
B
C
TIME (hr)

cultures. However, in contrast to the production of haemolytic activity found under normal growth conditions, stage C cultures rapidly lost haemolytic activity after maximum levels had been reached. A 10-fold increase in inoculum size in stage A caused an increase in β-haemolytic activity and supernatant activity at the start of stage C but maximum yields were not increased; altering the concentration of glucose added at the end of stage B from 0.05% to 2.0% (w/v) had a similar effect.

The effect of various carbohydrates on β- and supernatant haemolysin production using the stage-culture method was investigated. Stage A cultures were grown using 0.2% (w/v) glucose as carbon source. At the end of stage B various carbohydrates were added to the starved cells. Haemolytic activity was observed in stage C cultures with all the sugars listed in Table 14. Fructose, arabinose, mannitol and sorbitol yielded levels of haemolytic activity equal to or greater than that obtained with glucose-grown cultures. As pointed out by Rennie and Arbuthnott carry-over of residual glucose from stage A cultures, as monitored by the Nelson and Anthrone Methods (see page 61) did not occur.

Despite success in obtaining high yields of β-haemolysin and supernatant haemolytic activity in cultures grown by the three-stage method in CDM, passage of supernatant fluids through Millipore filters resulted, in most experiments, in complete loss of haemolytic activity. Also, haemolytic titrations using supernatant fluids or suspensions of E. coli cells required 3 hr to reach an end-point. In order to determine if there were viable organisms remaining in supernatant fluids after centrifugation, which either contributed to or were entirely responsible for haemolysis, supernatant fluid from stage C cultures was centrifuged at progressively higher speeds (Table 15). After each centrifugation the top half of fluid in the tubes was carefully removed with a needle and syringe; the remainder was discarded.

Table 14: The effect of various carbohydrates on production of β-haemolysin in chemically-defined medium.

Carbohydrate added	Growth[a]	Per cent haemolytic activity[a]	
		Supernatant	β-haemolysin (cells)
Glucose	100	100	100
Fructose	78	400	200
Lactose	47.5	25	25
Sucrose	0	12.5	25
Mannose	65	25	50
Maltose	30.6	25	50
Galactose	27.3	12.5	50
Arabinose	37.2	200	100
Mannitol	87	200	100
Sorbitol	54.3	200	100

[a] Growth (3 hr, stage C) and haemolytic activities (maximum) were measured as a percentage of glucose-grown cultures.

Table 15 : The effect on haemolytic activity of viable organisms remaining in supernatant fluids.

Supernatant Preparation	Procedure	Viable Count[1.] (cells/ml)	Haemolytic Activity[2.] (HU_{50}/ml)	
			Supernatant	Filtrate
I	Supernatant from whole culture fluid centrifuged at 10,000 g for 20 min.	1.7×10^7	128	< 2
II	Supernatant I centrifuged at 10,000 g for 20 min.	7.5×10^5	16	< 2
III	Supernatant II centrifuged at 16,000 g for 20 min.	1.0×10^5	4	< 2
IV	Supernatant III centrifuged at 25,000 g for 20 min.	3.7×10^3	< 2	< 2

1. The viable count from the whole culture fluid was 1.7×10^9 cells/ml.

2. The haemolytic activity of the cell pellet was 512 HU_{50}/ml.

Table 16 : Summary of the properties of β-haemolysin from E. coli strain 25238 grown in CDM.

Treatment	Effect on β-Haemolysin
Heat : 1 hr @ 56°C	Complete loss of activity.
Storage : 4°C	90 - 100% loss of activity after 48 hr.
Addition of bacteriostatic or bacteriocidal agents to titration diluent.	Activity inhibited by streptomycin (200 µg/ml), 0.001% (w/v) thiomersalate, and 0.5% (v/v) formaldehyde.
Removal of calcium ions from diluent	No activity unless calcium ions present in titration diluent.
Incubation at 37°C with trypsin (200 µg/ml)	Activity disappeared for 15 - 20 min, then reappeared and gave better activity than untreated preparations.

Haemolytic activity was determined and viable counts were made on each supernatant. In addition, samples were filtered and the haemolytic activity of the filtrates was monitored. From Table 15 it can be seen that, although a single centrifugation of whole culture fluid removed at least 99% of viable organisms, a comparatively large number remained in the supernatant fluid. Further centrifugation reduced the numbers of viable cells in supernatants but also caused a progressive loss of haemolytic activity. It is important to note that none of the filtrates obtained from these fluids were haemolytic. Furthermore, a sample of supernatant fluid I, kept at 4^{o}C until centrifugation procedures were completed, did not lose activity. The results of these experiments support the suggestion that supernatant haemolytic activity from cultures grown in CDM was due entirely to cell-associated or β-haemolysin.

2. Properties of β-haemolysin.

The properties of β-haemolysin were investigated and the results are recorded in Table 16. Cells from 1 hr, stage C cultures, suspended in saline to contain approximately 5×10^{8} viable cells/ml, were used for these studies. In addition, experiments were performed which showed that use of Veronal buffer, pH 7.3 (see Appendix II), as a diluent for titrations gave a sharper haemolytic end-point than 0.01 M borate buffer, pH 7.2, or 0.85% (w/v) saline to which was added 1% (w/v) peptone or BSA (1 mg/ml). All diluents tested contained 0.01 M calcium chloride (w/v).

Haemolysis did not occur unless calcium chloride was included in the titration diluent. When streptomycin sulphate (200 μg/ml) or 0.001% (w/v) thiomersalate were included in the diluent no haemolysis occurred; storage in 0.5% (v/v) formaldehyde for 10 hr at 4^{o}C also abolished β-haemolytic activity. The effect of trypsin on β-haemolysin is interesting. Trypsin-treated cell-suspensions became turbid after 20 - 30 min incubation at 37^{o}C;

at the same time β-haemolytic activity reappeared and increased to levels greater than found in untreated preparations. However, no haemolytic activity was observed in Millipore-filtered samples of trypsin-treated cell suspensions. Reappearance of haemolytic activity probably resulted from growth of E. coli in the presence of trypsin.

3. Removal of β-Haemolysin from viable E. coli cells.

Attempts to extract β-haemolysin from viable cells by gentle or vigorous shaking with glass beads or disruption of cells in an MSE 100 watt ultrasonic disintegrator were unsuccessful. Supernatant fluids and cell pellets obtained after these procedures contained no haemolytic activity.

The methods of Ginsburg, Bentwich and Harris (1965) and Ginsburg and Harris (1965), devised for isolation of the cell-bound form of streptolysin S, were performed with E. coli β-haemolysin. Two procedures were used:

1. E. coli cell suspensions were incubated with 'carrier' compounds which, in the case of streptolysin S, combine with the cell-bound haemolysin and result in its removal from cells (Ginsburg, Bentwich and Harris, 1965).

2. E. coli cells, after incubation with erythrocytes, were removed from the reaction mixture using DEAE cellulose columns. This was done in order to see whether cell-associated β-haemolysin adsorbed to erythrocytes (Ginsburg and Harris, 1965).

For the first method, a series of cultures were grown to the end of stage B. At this point, the cells were removed by centrifugation and were resuspended in flasks containing CDM with 50% (v/v) fresh human serum in distilled water, 5 mg/ml Tween 80 (Koch-Light, Colnbrook, England), 30 mg/ml BSA, fraction V (Armour Pharmaceuticals, England) or 5 mg/ml of ribonuclease-resistant, RNA core fraction (Worthington Biochemicals Ltd., U.S.A.). The

flasks were incubated at 37°C, 150 rev/min; samples were removed at 0.5 and 1 hr intervals and the haemolytic activity of Millipore-filtered supernatants was determined. Haemolytic activity (8 - 16 HU_{50}/ml) was observed only in filtrates of cultures grown in CDM containing BSA. The β-haemolytic activity of these cultures was 500 HU_{50}/ml.

The experimental procedure used for the second method is described on page 45 and outlined in Figure 2. Table 17 shows that passage of cell-erythrocyte mixtures through standardised DEAE cellulose columns removed a large proportion, but not all, of the viable organisms (sample time II). A most interesting observation was that, after interaction of viable E. coli cells and red cells, addition of thiomersalate to column effluents (fraction 1) did not inhibit subsequent haemolysis. Furthermore, viable cells were not found at the end of the experiment in thiomersalate-treated effluents (sample time III). This suggested that β-haemolysin was adsorbed to the erythrocytes. In the absence of thiomersalate (fraction 2), haemolytic activity increased between sample times II and III, but the numbers of viable cells also increased.

C. Production of α-Haemolysin.

An essential pre-requisite for further studies of the chemical nature and biological properties of E. coli haemolysin was the ability to obtain haemolytic activity in culture filtrates; viable cells containing haemolytic activity were unsatisfactory since activity was quickly lost during storage and moreover, as mentioned, only small amounts of α- or filterable haemolytic activity could be extracted from viable cells grown in CDM.

Therefore, a number of different complex liquid media were tested in attempts to demonstrate production of α-haemolysin. From Table 18, it

Table 17 : Interaction of β-haemolysin with sheep erythrocytes and removal of viable organisms

Time (min) prior to DEAE Cellulose Chromatography	% Increase in Haemolysis following DEAE Chromatography[a.]		Viable count (cells/ml x 10^2)[b.] of Fractions at Sample Time			
			II		III	
	Fraction 1[c.]	Fraction 2	1	2	1	2
0	< 1	< 1	1.3	1.1	0	1.7
20	21	14	1.7	1.9	0	9.0
40	30	19	3.9	2.4	0	12.0
60	5	2	4.0	4.4	0	18.0
60 (SRBC control)	0	0	0	0	0	0

a.) % haemolysis at sample time III minus % haemolysis at sample time II (see figure 2)

b.) Viable count in initial mixture was 1.7×10^7 E. coli cells/ml (sample time I)

c.) Fraction 1; 0.001% (w/v) thiomersalate added: Fraction 2; no thiomersalate added (see Materials and Methods, page 45)

Table 18 : Production of α-haemolysin in various media by strain 25238.

Medium[1].	Maximum Activities (HU/ml)		
	Cells (β-Haemolysin)	Supernatant	Filtrate (α-Haemolysin)
Chemically defined (CDM) (Snyder and Koch, 1966)	4000	100	0
Beef heart for infusion (Difco)	100	60	0
Fresh beef heart infusion (MEB) (Smith, 1963)	1200	300	300
Brain heart infusion (Difco)	2000	250	10
Yeast extract, casein hydrolysate (Bernheimer and Schwartz, 1963, modified by McNiven, 1972)	0	0	0
Nutrient broth (NBG) (Oxoid No. 2)	1300	3000	3000
Tryptone soya broth (Oxoid)	650	150	0

1. α-D-glucose was added to all media at a concentration of 0.2% (w/v).

can be seen that only MEB and NBG supported satisfactory production of α-haemolysin. Difco Brain Heart Infusion broth allowed elaboration of small amounts of α-haemolysin but, it is evident that, in this medium, most of the activity contained in supernatants was due to β-haemolysin. By contrast, comparison of supernatant and filtrate activities of cultures grown in MEB or NBG indicated that viable organisms remaining in supernatants did not significantly affect the haemolytic titre.

Six haemolytic strains from the hospital survey, including strain 25238, were tested in NBG medium to compare amounts of α-haemolysin produced within 2 - 3 hr (see Figure 6). Two strains elaborated α-haemolysin in amounts equivalent to strain 25238; two strains elaborated one-third to one-half as much α-haemolysin. By contrast, using strain 15370, no haemolysin appeared in culture filtrates, even though filtrates were assayed to 24 hr after inoculation. All strains produced similar amounts of β-haemolysin after 2 hr incubation.

It must be stressed here that, in order to confirm the sterility of culture filtrates, all filtered samples of supernatant fluids were checked carefully by plating aliquots of filtrates on to nutrient agar and MacConkey agar medium.

A typical example of growth and haemolysin production by strain 25238 in NBG medium is shown in Figure 6. The α-haemolysin was elaborated rapidly; maximum titres were reached within 2 hr after inoculation during the logarithmic phase of growth. In the case of β-haemolysin, significant amounts were found at 0 hr. Activity was highest between 1 and 2 hr and thereafter declined rapidly; no detectable β-haemolysin was found at 4 hr. This rate of disappearance was considerably greater than for α-haemolysin.

Less α-haemolysin was found in static than in shaken cultures.

Figure 6 : Production of α- and β-haemolysin in NBG medium by strain 25238

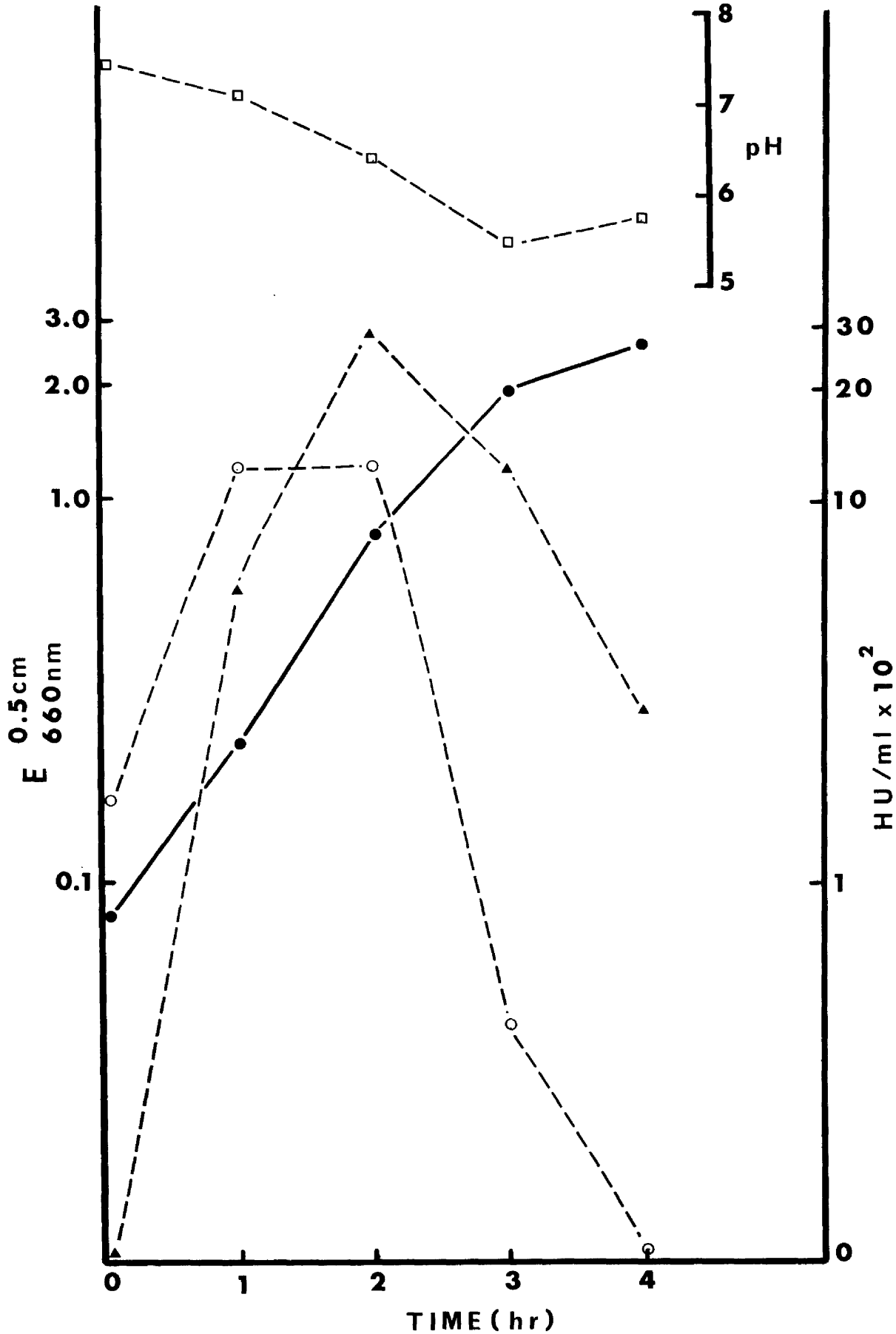
Symbols:

⊗ : Growth as $E^{0.5\ cm}_{660\ nm}$

△ : α-haemolysin (HU/ml x 10^2)

○ : β-haemolysin (HU/ml x 10^2)

□ : pH of culture filtrates


8
7
pH
6
5
3.0
2.0
1.0
0.1
E 0.5cm 660nm
30
20
10
1
0
HU/ml x 10²
0
1
2
3
4
TIME (hr)

Also, omission of glucose from the medium led to a 70% reduction in the amount of α-haemolysin found. Increasing the size of the initial inoculum caused progressive increases in maximum titres of α-haemolysin (Figure 7). The disappearance of α-haemolytic activity between 2 and 4 hr was not observed when a small inoculum was used. However the maximum titre was considerably lower than when larger inocula were employed. Routinely, an inoculum of $E_{660\ nm}^{0.5\ cm} = 0.08 - 0.10$ was selected and haemolysin was harvested at 2 hr.

Since previous reports had indicated that non-dialysable factors contained in peptone, egg white and ovalbumin enhanced production of α-haemolysin (see page 24) it was desirable to determine if certain factors contained in nutrient broth were responsible for production of α-haemolysin. For these studies sterile nutrient broth was passed under 20 lb/in^2 positive N_2 pressure through an Amicon ultrafiltration cell using a 'Diaflo' XM100A membrane which is reported by the manufacturers to retain macromolecules with molecular weights in excess of 1×10^5. The retained fluid and filtrate obtained by this procedure were adjusted to the same volume and concentration of Lowry positive material. When inoculated with strain 25238 the rate of growth was the same in each medium but the retained fluid enhanced production of α-haemolysin to levels greater than obtained with complete NBG medium (Figure 8). By contrast, the filtrate only supported production of small amounts of α-haemolysin.

D. Purification of α-Haemolysin.

1. Stage I: Concentration by precipitation with ammonium sulphate.

Preliminary pilot experiments showed that addition of solid ammonium sulphate to crude culture filtrate until 50% (w/v) saturation was reached precipitated all the haemolytic activity. No precipitation occurred

Figure 7 : The effect of inoculum size on production of α-haemolysin

Symbols:

◎ : Growth with initial inoculum size $E^{0.5\ cm}_{660\ nm} = 0.02$

▲ : Growth with initial inoculum size $E^{0.5\ cm}_{660\ nm} = 0.04$

○ : Growth with initial inoculum size $E^{0.5\ cm}_{660\ nm} = 0.10$

◎ : α-haemolysin (HU) produced from initial inoculum size $E^{0.5\ cm}_{660\ nm} = 0.02$

▲ : α-haemolysin (HU) produced from initial inoculum size $E^{0.5\ cm}_{660\ nm} = 0.04$

○ : α-haemolysin (HU) produced from initial inoculum size $E^{0.5\ cm}_{660\ nm} = 0.10$

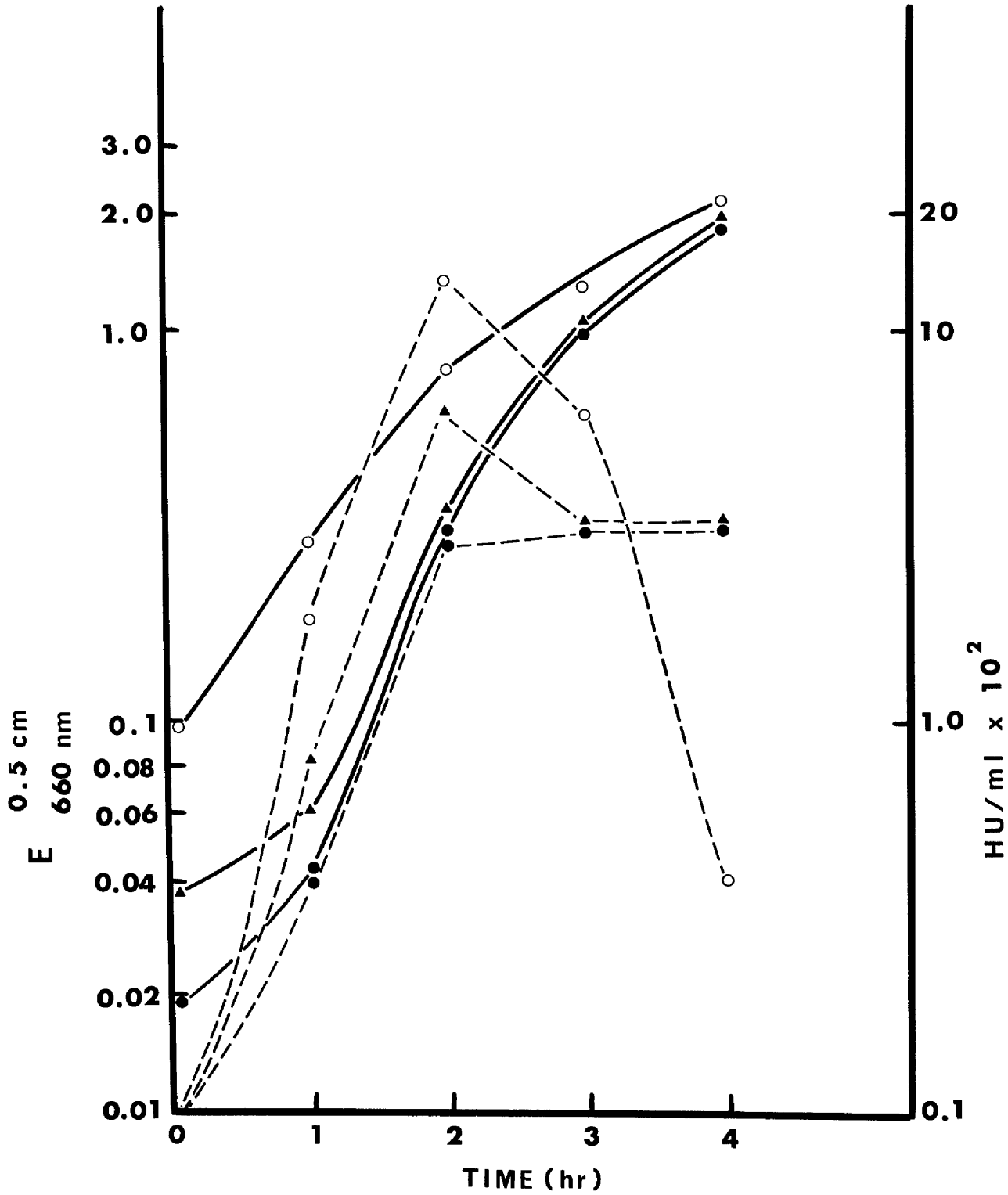

3.0
2.0
1.0
0.1
0.08
0.06
0.04
0.02
0.01
E 0.5 cm 660 nm
20
10
1.0
0.1
HU/ml x 10²
0
1
2
3
4
TIME (hr)

Figure 8 : The effect of ultrafiltered components of nutrient broth medium on α-haemolysin production

Symbols:

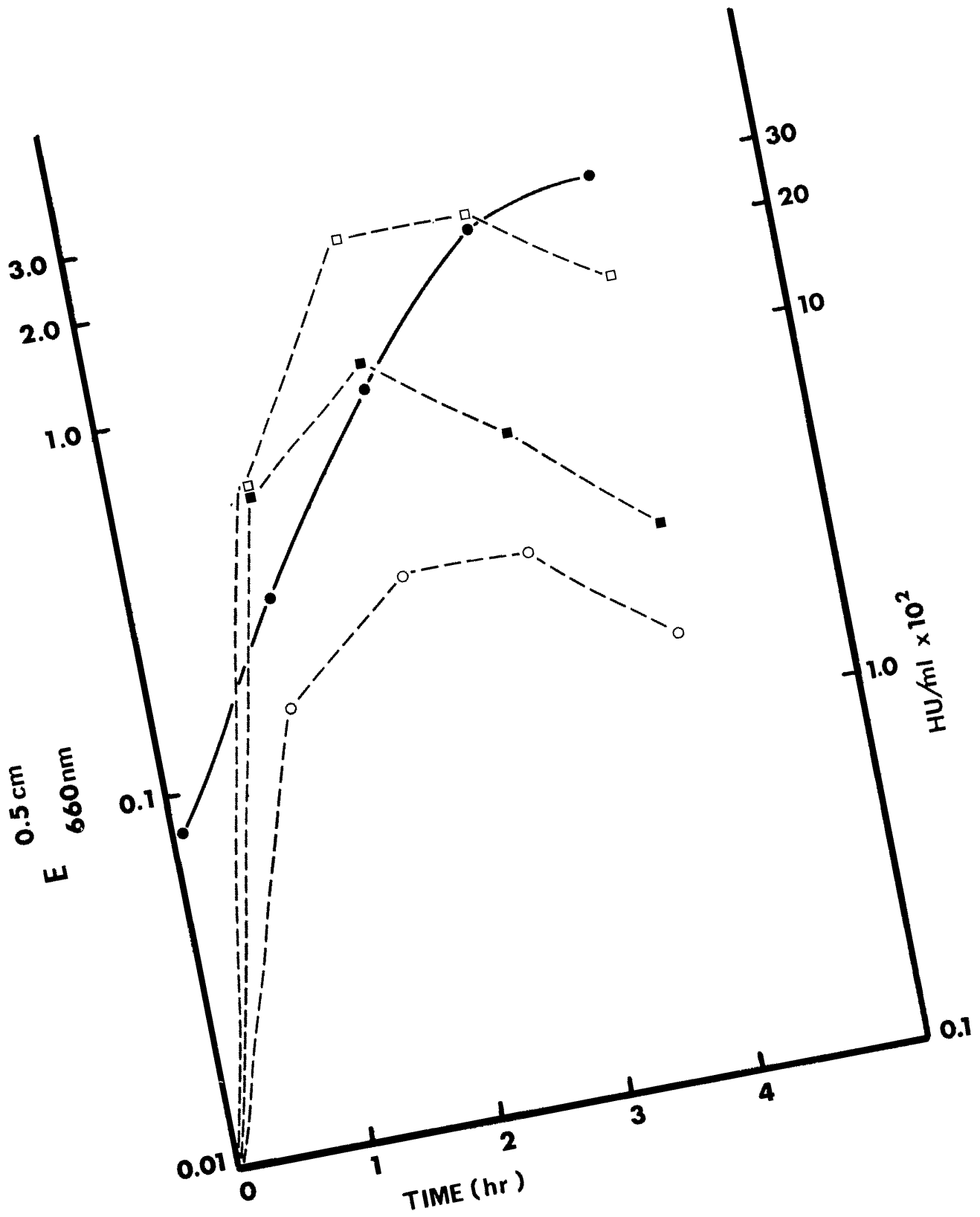
⊗ : growth as $E^{0.5\ cm}_{660\ nm}$ (the same in all media).

○ : production of α-haemolysin in filtrate after nutrient broth was passed through a 'Diaflo' XM100A membrane.

▣ : production of α-haemolysin in complete NBG medium.

□ : production of α-haemolysin in fluid retained after passing nutrient broth through a 'Diaflo' XM100A membrane.

All media contained 0.2% (w/v) α-D-glucose.


3.0
2.0
1.0
0.1
0.01
0.5 cm
E 660nm
0
1
2
3
4
TIME (hr)
30
20
10
1.0
0.1
HU/ml x10²

until 30 - 35% saturation with ammonium sulphate was achieved. Concentration of large volumes of culture filtrate by this method had 2 major advantages:

1. Precipitation of α-haemolysin was complete within 18 hr at $4^{o}C$.
2. Removal of as yet unidentified medium components appeared to activate α-haemolysin such that total recovered activity was greater than 100%.

No loss of haemolytic activity was found after dialysis of ammonium sulphate precipitates against distilled water adjusted to pH 8.0 with 0.5N NaOH. Use of distilled water at alkaline pH was used routinely because it was noted that precipitation of re-dissolved ammonium sulphate precipitates occurred occasionally if untreated distilled water (which varied between pH 5.0 and 6.0) was employed for dialysis.

2. Electrofocusing studies.

The technique of iso-electric focusing was investigated as a practical method of purifying stage I α-haemolysin. The results of electro-focusing a sample of α-haemolysin in a broad pH 3 - 10 gradient are shown in Figure 9. Although a high percentage (70%) of the total haemolytic activity applied to the column was recovered in these experiments as two closely associated peaks of activity with pI's of 4.6 and 5.0, it can be seen from Figure 9 (horizontal bar) that over 80% of haemolytic activity precipitated in the gradient. In addition, it was found that some medium components precipitated at acid pH. The major problem experienced was that, during removal of fractions from the column, precipitated haemolysin and medium components occasionally adhered to the sides of the column and re-dissolved at higher pH. It was not possible to control this effect and the component having a pI = 5.0 is likely to be an artifact.

Figure 9: Electrofocusing of stage I α-haemolysin in a broad pH gradient

Electrofocusing of stage I α-haemolysin prepared from strain 25238 culture filtrate. 450 mg of haemolysin were applied to the LKB 8101 column in a pH 3-10 gradient. Electrofocusing was carried out for 48 hr at $4^{\circ}C$ with a final potential of 800 V.

Fractions of 2 ml were collected.

Symbols:

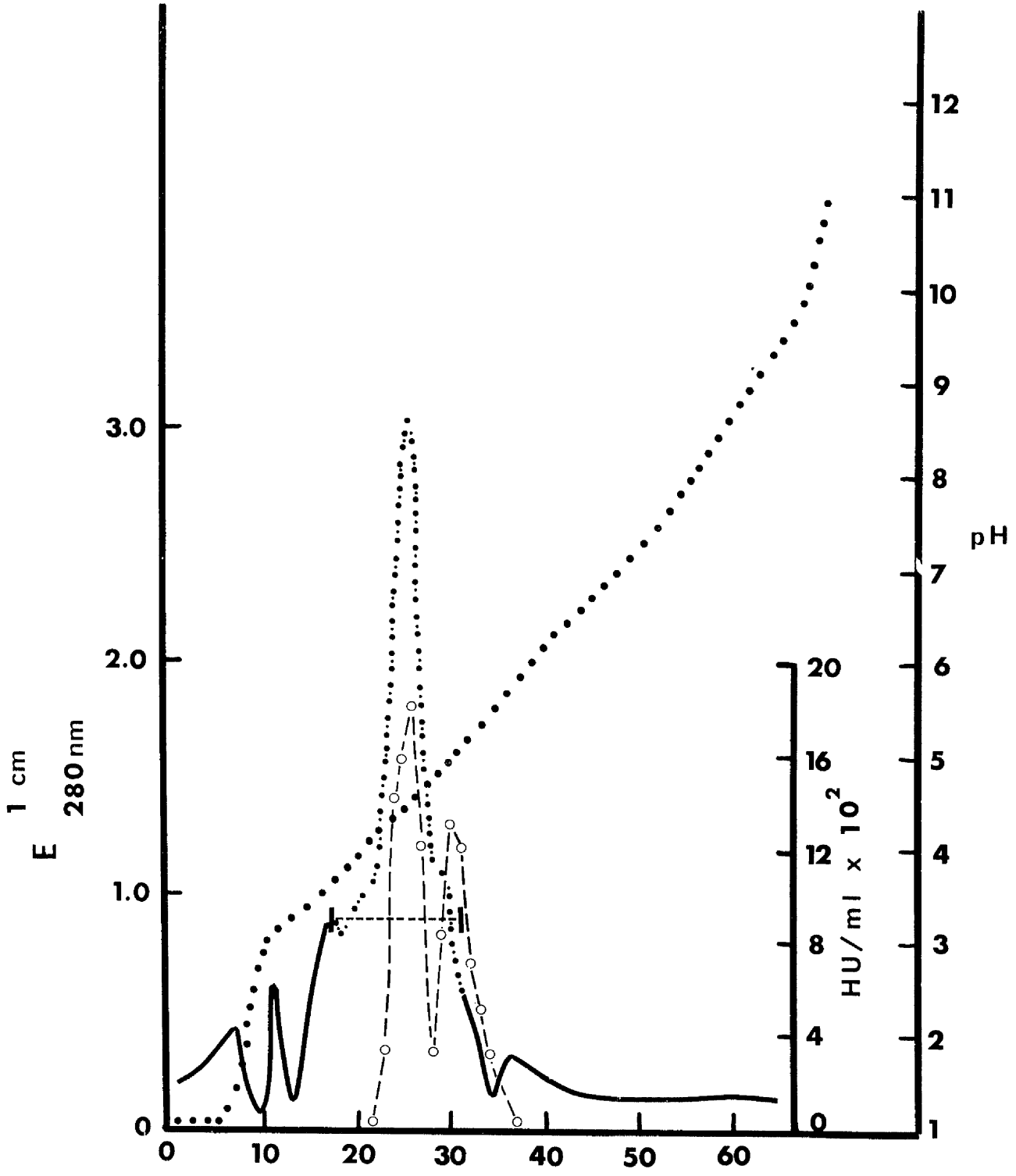
⊛ : pH of column fractions.

——— : $E^{1\ cm}_{280\ nm}$ of non-precipitated fractions.

|------| : Fractions showing precipitation (17-31).

•••••• : $E^{1\ cm}_{280\ nm}$ of precipitated fractions; precipitates first removed by centrifugation and re-dissolved in Veronal buffer, pH 7.3 before $E_{280\ nm}$ was measured.

o— — o : α-haemolysin (HU/ml x 10^2).


E 1 cm 280 nm
3.0
2.0
1.0
0
0
10
20
30
40
50
60
HU/ml x 10²
20
16
12
8
4
0
pH
12
11
10
9
8
7
6
5
4
3
2
1

Attempts were made to prevent precipitation of α-haemolysin at its iso-electric point by incorporation of urea in the system. Preliminary experiments were performed to find a suitable concentration of urea which would prevent precipitation but would not affect substantially the activity of α-haemolysin. For these studies, samples of stage I α-haemolysin were dialysed against distilled water adjusted to pH 4.0 with 1N HCl. The precipitates which formed were re-dissolved in distilled water containing 0 - 6M urea. To 1 ml of sample in urea was added 4.25 ml of an equimolar concentration of urea and 0.25 ml of 10% (v/v) pH 3 - 6 ampholine which was also in urea. The volumes used were scaled down 10-fold from the actual volumes employed in the light solution for a 110 ml column. After 24 hr at 4°C, the preparations were examined for precipitation.

Figure 10 shows that even low concentrations of urea markedly affected haemolytic activity. A concentration of 3.5M urea was required to reduce precipitation in the presence of ampholine to a minimum but, at this concentration of urea, haemolytic activity was reduced in 24 hr by more than 50%. At higher concentrations of urea precipitation was prevented completely, but over 90% of the original activity was lost and furthermore, could not be recovered after removal of urea by extensive dialysis. In samples treated with 0.4% (w/v) Triton X (Koch-Light, Colnbrook, England) in the presence of ampholine, α-haemolysin was found only in precipitates.

Despite initial high loss of activity in urea, stage I α-haemolysin, prepared by the procedure described above was electrofocused in a narrow pH 3 - 6 gradient, which contained 3.5M urea in the light and heavy solutions (see Appendix III). In this system haemolytic activity was resolved into 3 poorly defined peaks, 2 minor ones with pI's of 4.4 and 4.9 and a major peak with a pI of 4.7 (Figure 11) which was similar to the pI of the major peak found in a broad gradient system. Nevertheless, precipitation in this region of the gradient (horizontal bar) occurred before

Figure 10 : The effect of urea on solubility and stability of α-haemolysin in the presence of ampholine (pH range 3-6)

Samples of stage I α-haemolysin were precipitated by dialysis against distilled water adjusted to pH 4.0 with HCl. The precipitates were re-dissolved in 0-6M urea containing 0.45% (v/v) pH 3-6 ampholine. After storage for 24 hr at $4^{o}C$, α-haemolytic activity was determined.

Symbols:

columns represent α-haemolysin activity (HU/ml x 10^{3})

- : no precipitation

+/- : slight precipitation

+,++,+++ : increasing amounts of precipitate

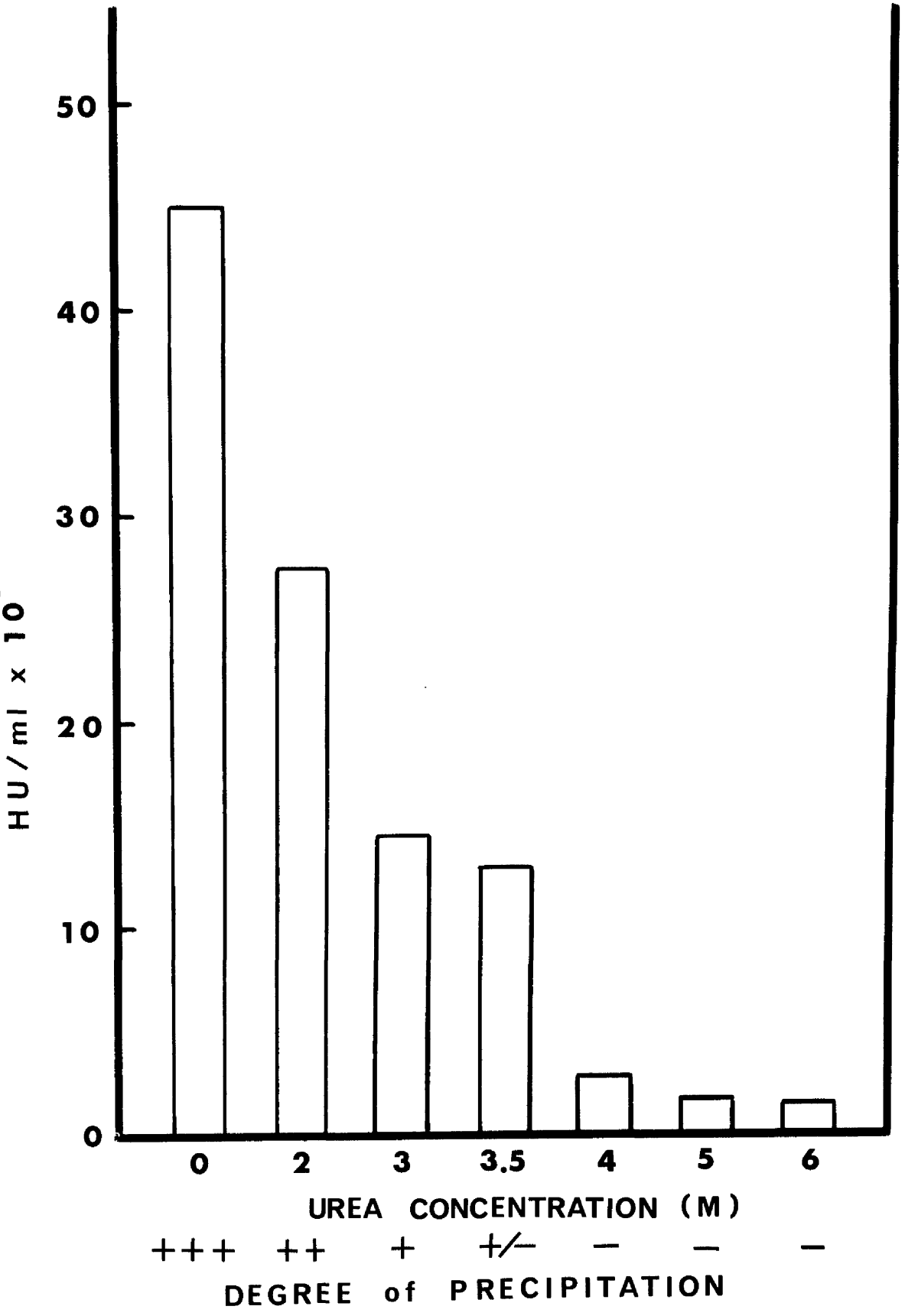

50
40
30
20
10
0
HU / ml x 10^3
0
2
3
3.5
4
5
6
UREA CONCENTRATION (M)
+++
++
+
+/−
−
−
−
DEGREE of PRECIPITATION

Figure 11: Electrofocusing of stage I α-haemolysin in a narrow pH gradient containing 3.5 M urea

Electrofocusing of stage I α-haemolysin prepared from strain 25238 culture filtrate. 40 mg of a precipitate, obtained by dialysis of stage I α-haemolysin against distilled water adjusted to pH 4.0 and re-dissolved in 3.5M urea, was applied to the LKB 8101 column in a pH 3-6 gradient containing 3.5M urea. Electrofocusing was carried out for 36 hr at 4°C with a final potential of 900 V. Fractions of 2 ml were collected.

Symbols:

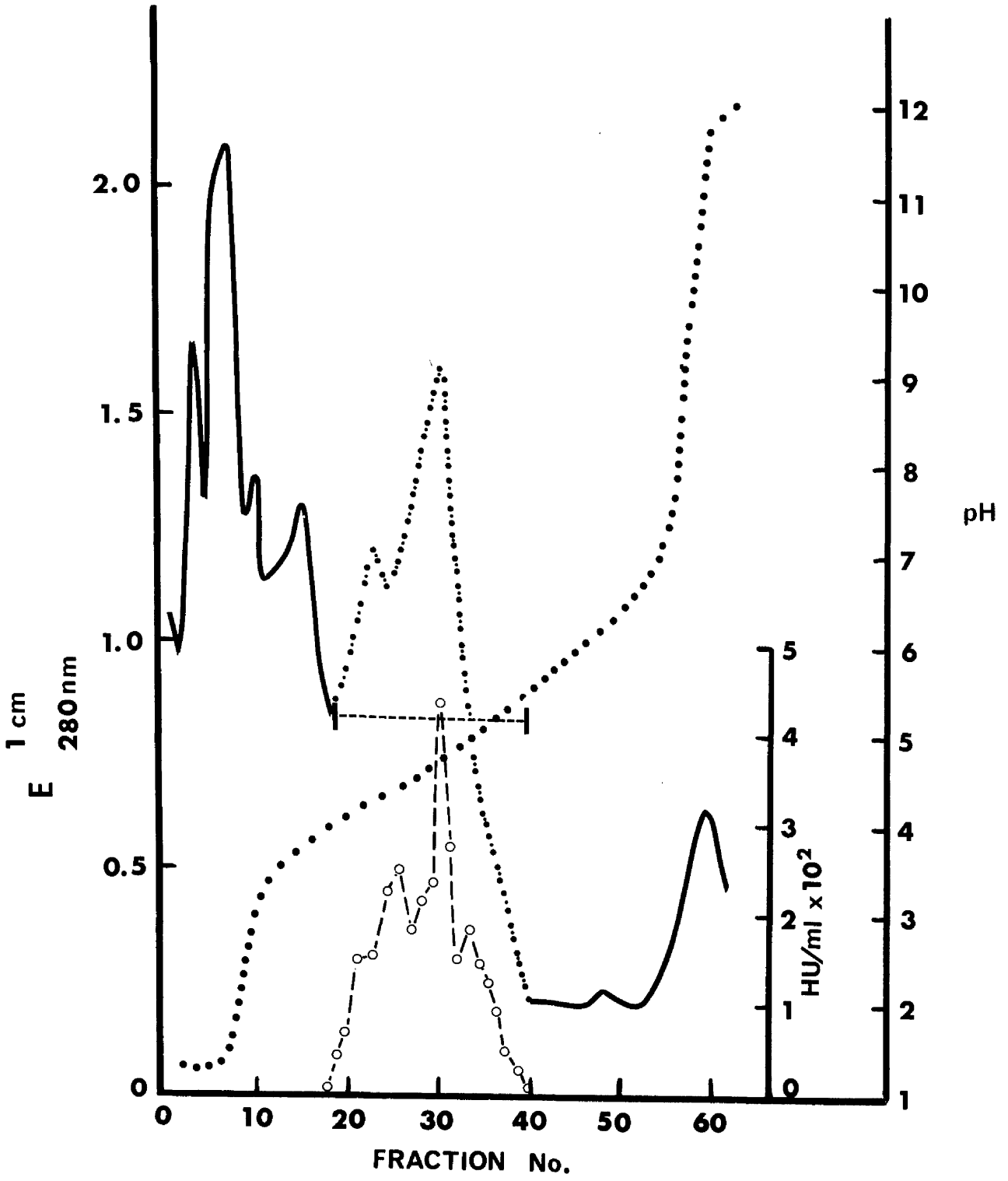
⊙ : pH of column fractions

——— : $E_{280\ nm}^{1\ cm}$ of non-precipitated fractions

|------| : Fractions showing precipitation (20-40)

•••••• : $E_{280\ nm}^{1\ cm}$ of precipitated fractions; precipitate first removed by centrifugation and re-dissolved in Veronal buffer, pH 7.3, before $E_{280\ nm}$ was measured.

o — — o : α-haemolysin (HU/ml x 10^2)


E 1 cm 280 nm
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
FRACTION No.
HU/ml x 10²
5
4
3
2
1
0
12
11
10
9
8
7
6
5
4
3
2
1
pH

electrofocusing was complete. Also, only 1 - 2% of the starting activity was recovered from the column.

Although electrofocusing proved to be unsuitable for purification of α-haemolysin, information regarding solubility properties of α-haemolysin at its iso-electric point gained by this technique was valuable for further purification procedures.

3. Stage II: Selective precipitation in acetate buffer.

Pilot experiments were performed to determine if stage I α-haemolysin could be precipitated selectively at its iso-electric point. A sample of α-haemolysin was dialysed at 4°C against 2 or 3 changes of 0.005M acetate buffer, pH 4.3 until precipitation was complete. The precipitate was collected by centrifugation, dissolved in Veronal buffer, pH 7.3 to the original sample volume and 0.1 ml was removed for haemolytic titration. The dialysis procedure was repeated using 0.005M acetate buffer adjusted to successively higher pH values. Supernatant fluids and precipitates re-dissolved in Veronal buffer were titrated for haemolytic activity.

Table 19 shows that, after dialysis against acetate buffer at pH 4.6, no measureable haemolytic activity remained in the supernatant fluid. When compared to untreated stage I α-haemolysin it can be seen that dialysis at pHs above the iso-electric point led to loss of α-haemolysin. Precipitation of α-haemolysin at pH 4.6 not only reduced the content of Lowry-positive material considerably (from 20 - 25 mg/ml to 3 - 4 mg/ml) but also had an activating effect on α-haemolysin similar to that observed after precipitation of culture filtrate with ammonium sulphate at 50% saturation. When this second precipitation procedure (stage II) was scaled up to deal with larger volumes of stage I α-haemolysin the activation effect was even more pronounced.

Table 19 : Stepwise precipitation of stage I α-haemolysin in 0.005M acetate buffers

Buffer pH	Supernatant Activity (HU/ml)	Activity in Precipitate (HU/ml)
4.3	40	31,000
4.6	< 20	38,000
4.8	200	14,000
5.0	4,000	13,000
Control : Stage I α-haemolysin	33,000	-

4. Stage III: Gel filtration on Sephadex G-200.

Preparation of the Sephadex and the apparatus has been described in detail (see Materials and Methods, section E.1). Initial fractionation of stage II α-haemolysin using 0.01M Tris-HCl, pH 7.3, as eluant buffer resulted in elution of a single peak of haemolytic activity at the void volume of the column. However, recovered activity was less than 2%. When 0.1M NaCl was added to the eluant buffer (i.e. TS buffer) recovered activity was increased 4 to 5 times and a slight shoulder appeared in the single peak of α-haemolysin (Figure 12).

As a possible method of stabilising α-haemolysin during gel filtration, 5% (v/v) glycerol was added to TS buffer (i.e. TSG buffer). This reasoning was based on the studies of Bernheimer and Grushoff (1967) who showed that the stability of cereolysin was increased during purification by addition of 5% (v/v) glycerol to haemolytic preparations. When stage II α-haemolysin was run in TSG buffer, 28 - 30% of the total activity applied to the column was recovered (Figure 13). In addition, α-haemolytic activity was eluted from the column at the void volume as two closely-associated but distinct peaks of activity (A and B). Peak fraction A corresponded to the single peak of activity seen in Figure 12. The separation of A and B was reproduced consistently in 3 runs with the same batch of stage II α-haemolysin and 2 runs using a different batch of stage II α-haemolysin.

The material which eluted between 200 and 500 ml was brown pigmented material which contained no detectable haemolytic activity. A similar elution profile was obtained when sterile nutrient broth, concentrated by precipitation with 50% (w/v) saturated ammonium sulphate and pH 4.6 acetate buffer, was run in TSG buffer. However, no haemolytic activity was found and the $E_{280\ nm}$ in the region between 120 and 180 ml of eluate was about one-fifth that observed with active α-haemolysin preparations.

Figure 12: Fractionation of stage II α-haemolysin on Sephadex G-200 using TS eluant buffer

A sample of 10 ml of stage II α-haemolysin (35 mg protein) was applied to a K26/100 Pharmacia column containing Sephadex G-200 (medium grade). The system was run in TS (0.01M Tris, 0.1M NaCl, pH 7.3) buffer at 4°C. The flow rate was 15 - 18 ml/hr. Fractions of 5 ml were collected after 100 ml of eluate had passed through the column.

Symbols:

——— : $E^{1\ cm}_{280\ nm}$

– – – : α-haemolysin (HU/ml x 10^3)

Vo : Eluate volume at which peak of Blue Dextran 2000 elutes.

Figure 13: Fractionation of stage II α-haemolysin on Sephadex G-200 using TSG eluant buffer

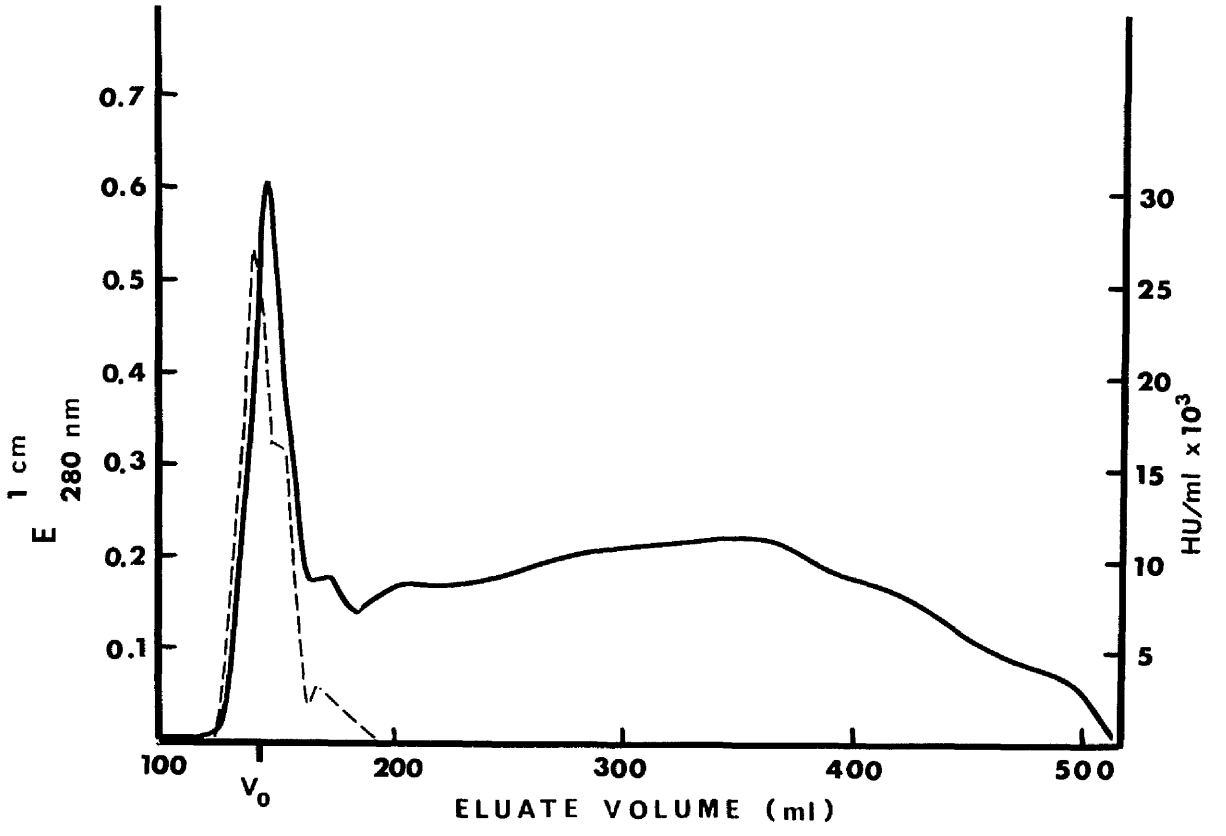
A sample of 10 ml of stage II α-haemolysin (35 mg protein) was applied to a Pharmacia K26/100 column containing Sephadex G-200 (medium grade). The system was run in TSG (0.01M Tris, 0.1M NaCl, 5% (v/v) glycerol) buffer at 4°C. The flow rate was 15 - 18 ml/hr. Fractions of 5 ml were collected after 100 ml of eluate had passed through the column.

Symbols:

——— : $E^{1\ cm}_{280\ nm}$

– – – : α-haemolysin (HU/ml x 10^4)

Vo : Eluate volume (ml) at which peak of Blue Dextran 2000 elutes.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
E 1 cm 280 nm
30
25
20
15
10
5
HU/ml x 10³
100
V₀
200
300
400
500
ELUATE VOLUME (ml)

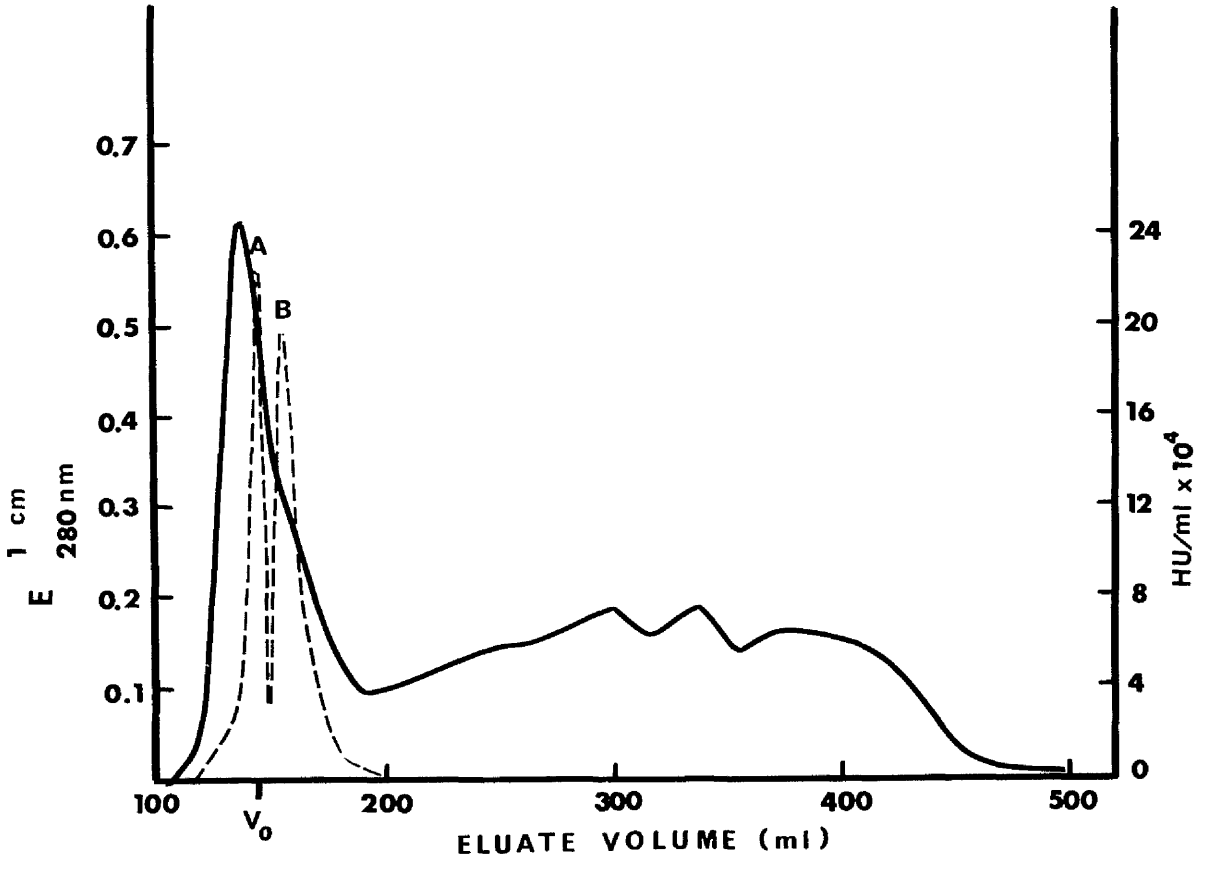

0.7
0.6
0.5
0.4
0.3
0.2
0.1
E 1 cm 280 nm
A
B
24
20
16
12
8
4
0
HU/ml x 10⁴
100
V₀
200
300
400
500
ELUATE VOLUME (ml)

Table 20 shows a summary of the purification of α-haemolysin in a typical experiment using stage I, stage II, and Sephadex G-200 chromatography (stage III). Indeed, specific activities using these procedures were very high and 76% of the total activity contained in crude culture filtrate (stage 0) was recovered from peak fractions A and B after Sephadex chromatography. However, it must be stressed that the final % recovery in peak fractions A and B after Sephadex G-200 fractionation (stage III) is compared only to the total activity contained in crude culture filtrate (stage 0). This does not take into account the fact that during purification both activation (stages I and II) and loss of α-haemolytic activity (stage III) occurred.

Amicon ultrafiltration of stage I and stage II α-haemolysin was used occasionally. During this time however, the 'Diaflo' XM100A membrane was the largest pore size filter available in the laboratory. Haemolytic activity contained in both stage I and stage II preparations was retained by this filter and was concentrated 5 to 10 times as monitored by haemolysis of sheep erythrocytes. However, pigmented components were concentrated also and no net purification was achieved.

E. Physico-Chemical Characteristics of α-Haemolysin.

1. Physical properties.

Stability: Samples of stage II α-haemolysin were heated for 30 min at 37°C, 61°C and 81°C; other samples were stored at 4°C, -20°C and -70°C for periods up to 2 weeks. Haemolytic titrations were performed and % reduction in α-haemolytic activity was recorded. An aliquot of stage III α-haemolysin was included to confirm results obtained with less pure preparations.

In these experiments, 50% reduction in haemolytic activity was observed in 30 min at 37°C; complete inactivation occurred at higher

Table 20 : Summary of the standard purification procedure

Stage of Purification	Vol (ml)	Total Activity (IU x 10^3)	Protein (mg/ml)	Specific Activity (IU/mg)	Increase in Specific Activity	Recovery[2.] %
0	1,750	5,250	11.6	260	-	-
I	125	18,750	19.7	7,600	29	357
II	85	72,250	3.6	236,000	907	1,376
III[1.]	10	4,000	0.4	1,000,000	3,850	76

1. Pooled activities of peak fractions A and B were used to determine specific activity

2. Recovered activity at each stage compared to activity in original culture filtrate (Stage 0)

temperatures. Even at 4°C, 50% of the original activity was lost after 24 hr. However, at -20°C and -70°C stage II preparations retained activity for long periods. Stage III haemolysin retained activity for 6 - 8 days at -20°C but progressively lost haemolytic activity thereafter. Other experiments showed that lyophilisation caused inactivation of α-haemolysin. No inhibition of haemolytic activity was observed if 0.001% thiomersalate was added to the titration diluent.

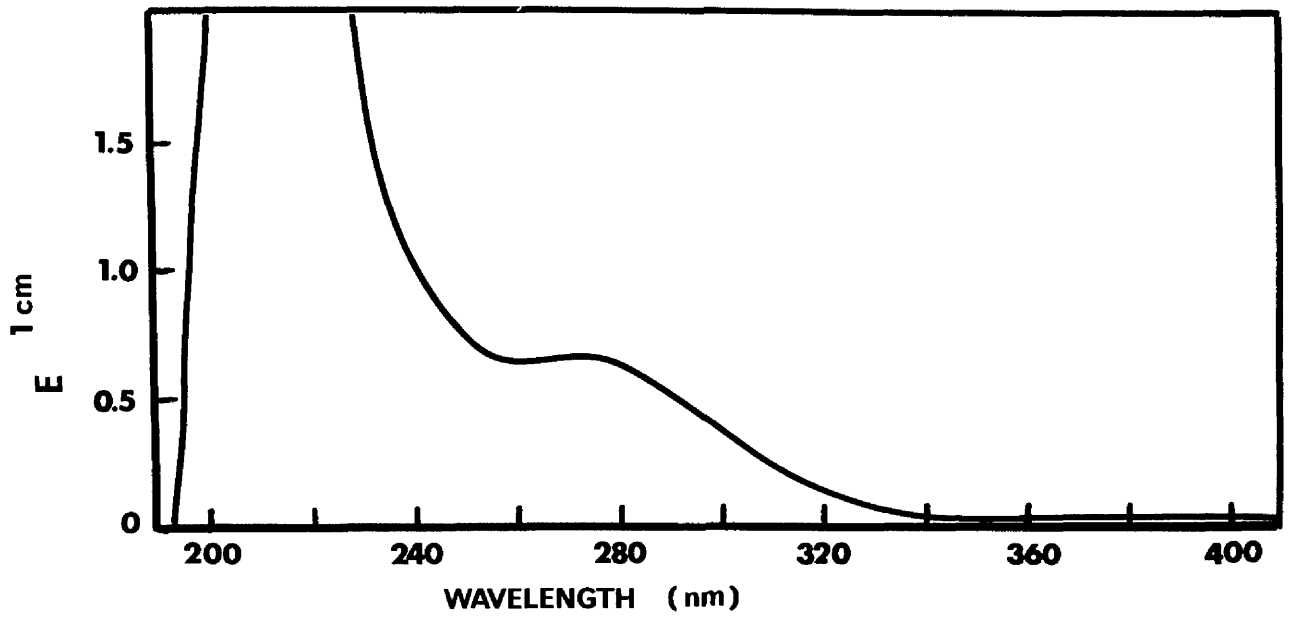
Effect of proteolytic enzymes: Trypsin (ex bovine pancreas; Koch-Light) and pronase (ex Streptomyces griseus; Koch-Light) were added at 200 μg/ml final concentration to samples of stage II α-haemolysin kept at room temperature. At intervals of 20, 40 and 60 min aliquots were removed and titrated. Both enzymes caused complete inactivation of α-haemolysin within 20 min whereas an untreated sample retained activity during the experiment.

Ultra-violet absorption spectrum: Figure 14 shows the U.V. absorption spectrum of stage III α-haemolysin (peak A). A shoulder, characteristic of protein-containing solutions, occurred at 276-280 nm with an additional peak of greater magnitude between 200 and 230 nm. Peak fraction B gave an equivalent U.V. absorption spectrum but the height of the peak at 280 nm, as expected, (see Figure 13) was much lower than that of peak fraction A. No absorption was observed in the visible light range with either preparation.

Immunodiffusion: Samples of stage I and stage II α-haemolysin, and samples of stage III purified fractions A and B concentrated by precipitation in 85% saturated ammonium sulphate, were dialysed for 6 hr at 4°C against 0.01M Tris, 0.1M potassium chloride, pH 7.2. Antiserum to stage II α-haemolysin, heat-inactivated for 30 min at 56°C and dialysed against Tris-potassium chloride buffer, was applied to the centre well of the microscope slide system described on page 59. Haemolysin preparations were applied to the surrounding wells. The advantages of this micro-immunodiffusion technique

Figure 14: Ultra-violet absorption spectrum of stage III α-haemolysin, peak fraction A

Peak fraction A, obtained by fractionation of stage II α-haemolysin on Sephadex G-200 using TSG eluant buffer (see Figure 13), was analysed for absorption in the spectrum of ultraviolet light. Note the shoulder at 280 nm characteristic of aromatic amino acids.


1.5
1.0
0.5
0
E 1 cm
200
240
280
320
360
400
WAVELENGTH (nm)

were that only minute quantities of antigen and antiserum were used, and in general a precipitin line developed within 24 hr.

A series of preliminary experiments indicated that undiluted antiserum gave the most distinct band of precipitation at the concentrations of antigen used and Plate I shows a major line of identity between the four samples of α-haemolysin tested. A faint precipitin line can be seen also in stage II and peak fraction A preparations which has only migrated slightly away from the antigen wells. This band was not found in the peak B preparation even though both A and B preparations were concentrated to contain the same amount of Lowry-positive material before application to the wells. In addition, the line of precipitation formed from fraction B was less intense than that formed from fraction A. No immuno-diffusion lines appeared when pre-immune antiserum was employed in the centre well.

Molecular weight studies: The finding that α-haemolysin was eluted from Sephadex G-200 at the void volume of the column (Figure 13) indicated that its molecular weight was at least 2×10^5. To obtain further information regarding the molecular size of α-haemolysin a sample of stage II α-haemolysin was passed through a 'Diaflo' XM100A ultrafilter. The retained fluid and filtrate were diluted in TS buffer to the same volume as the original sample and titrated for haemolytic activity. The preparation which contained haemolytic activity (i.e. the retained fluid) was then passed through an XM300 membrane and both retained fluid and filtrate were again diluted and titrated. The ultrafiltration cell purchased was too large to permit similar experiments to be performed with stage III α-haemolysin.

Table 21 shows that by this technique a rough approximation of the molecular size of α-haemolysin, between 1×10^5 and 3×10^5, was obtained. The 6% of α-haemolysin apparently retained by the XM300 filter could be accounted for by the fact that ultrafiltration was not continued until the contents of the cell were dry.

Plate I. Microslide immunodiffusion of α-haemolysin preparations

Symbols:

1. stage I haemolysin.
2. stage II haemolysin.
3. Sephadex G-200 peak A fraction.
4. Sephadex G-200 peak B fraction.

As. antiserum to stage II haemolysin.

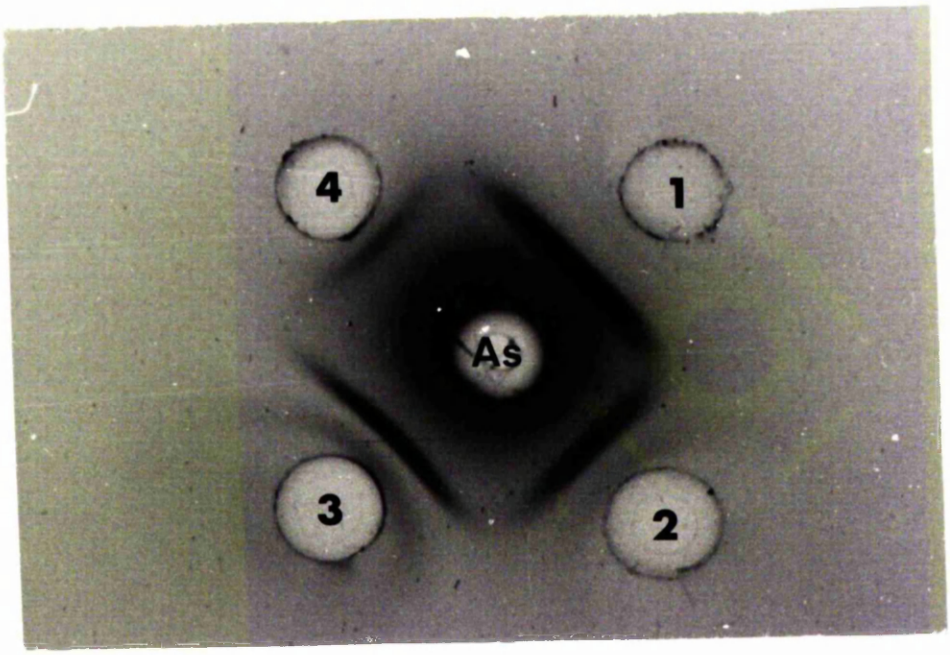

4
1
As
3
2

Table 21 : Estimation of the molecular size of α-haemolysin by 'Diaflo' membrane ultrafiltration.

Amicon 'Diaflo' Membrane	Approximate Exclusion Limit (M.W.)	Sample	% Total Activity
XM100A	1×10^5	Filtrate	0
		Retained fluid	100
XM300	3×10^5	Filtrate	94
		Retained fluid	6

Plate II. Estimation of diffusion coefficient (D_{20}) of purified α-haemolysin.

Symbols:

A_G : Stage III α-haemolysin (peak fraction A) concentrated 5 times and dialysed against 0.01M Tris - 0.1M KCl buffer, pH 7.2.

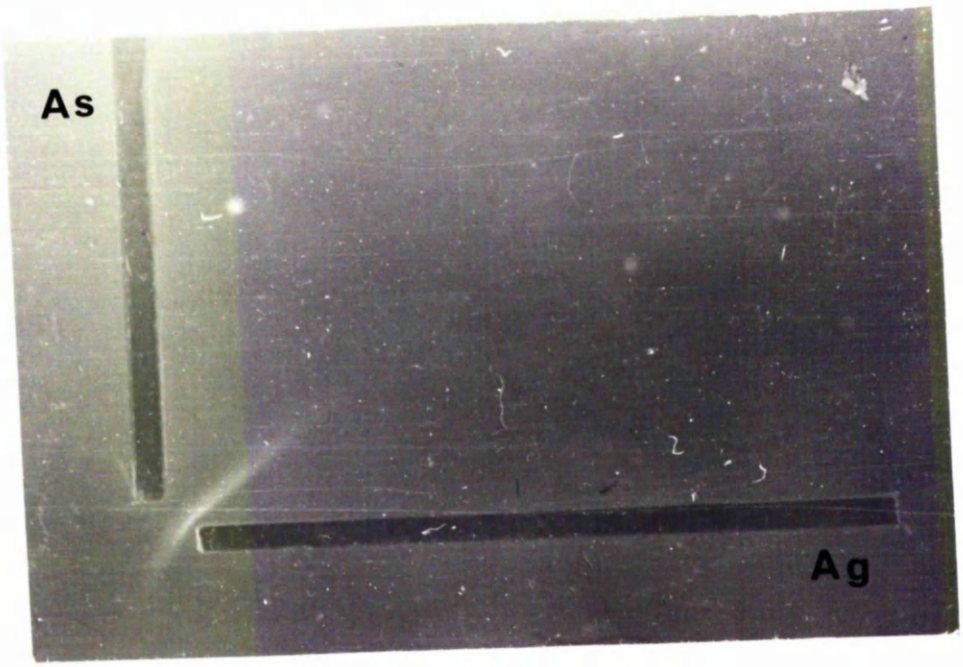
A_S : Antiserum to stage II α-haemolysin; undiluted.

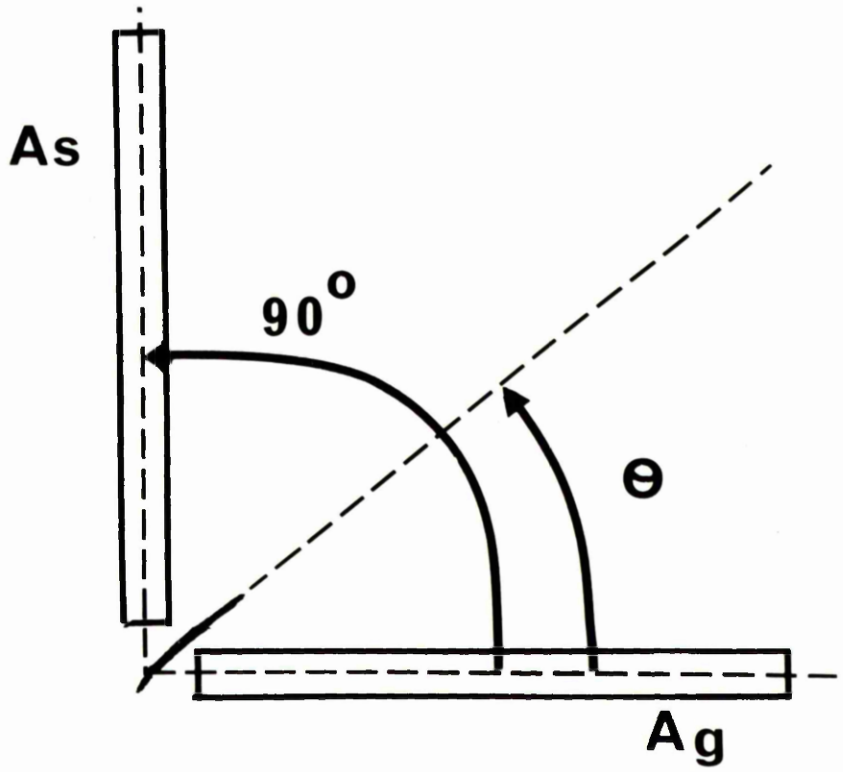
Figure 15. Schematic diagram showing measurement of the diffusion coefficient (D_{20}) of α-haemolysin.

Symbols:

A_G; A_S : Same as plate II above.

θ : Angle to 90° to which A_G diffuses according to its molecular size.


As
Ag


As
90°
Θ
Ag

Estimation of the diffusion coefficient (D_{20}) was used to determine an approximate molecular weight for α-haemolysin. Plate II shows the precipitin line obtained when stage III α-haemolysin was run against undiluted antiserum. On duplicate estimations of the angle θ (Figure 15), using a magnifying eyepiece with 0^{o} - 90^{o} markings in 1^{o} units, a value of $39^{o} \pm 0.5^{o}$ was obtained. The D_{20}, calculated according to the formula given on page 59 was 2.4×10^{-7} cm^{2} sec^{-1}.

Although the diffusion coefficients of a spectrum of standards (see Allison and Humphrey, 1960) were not determined, human serum albumin (HSA, fraction V; Sigma, London) was run against anti-HSA prepared in rabbits to confirm at least for HSA, that the system used in this thesis was comparable to that reported by Allison and Humphrey. The test sample of α-haemolysin was included on the same agar plate.

A value for θ of 52^{o} was obtained for HSA; α-haemolysin gave a value of 39.5^{o}. Interpolating these findings to the diffusion coefficients reported by Allison and Humphrey gave a value of 7×10^{4} for the molecular weight of HSA and 5 to 6×10^{5} for α-haemolysin. A more accurate estimation by this technique would require measurement of diffusion coefficients of a variety of large antigenic molecules of known molecular weight.

Disc gel electrophoresis in the presence of SDS can be used to determine molecular weights of purified proteins by comparison with standard proteins of known molecular weight run under the same conditions. Attempts were made to determine the molecular weight of purified α-haemolysin by this technique. The major problem encountered was failure of α-haemolysin to penetrate into the separating gel. Table 22 gives a summary of the conditions varied in order to achieve penetration of α-haemolysin into the gel columns. A small amount of peak fraction A did enter the separating gel

Table 22 : Summary of attempts to determine the molecular weight of α-haemolysin by SDS disc-gel electrophoresis

Condition varied	Effect on α-Haemolysin
Acrylamide concentration 3.5% - 11.6%	No penetration into the separating gel
SDS concentration 0.2% - 2%	No penetration into the separating gel
pH : 4.9 - 8.3	No penetration into the separating gel
1.6% (v/v) mercaptoethanol added	No penetration into the separating gel
6M urea, 8.3% (w/v) glycine and 2% SDS added to sample; No stacking gel used; 3.5% acrylamide in separating gel.	A small amount of sample penetrated into the separating gel; most of the sample remained at the top of the gel column.

Plate III. Urea-SDS disc-gel electrophoresis of α-haemolysin.

Symbols:

A: Stage III α-haemolysin (peak fraction A).

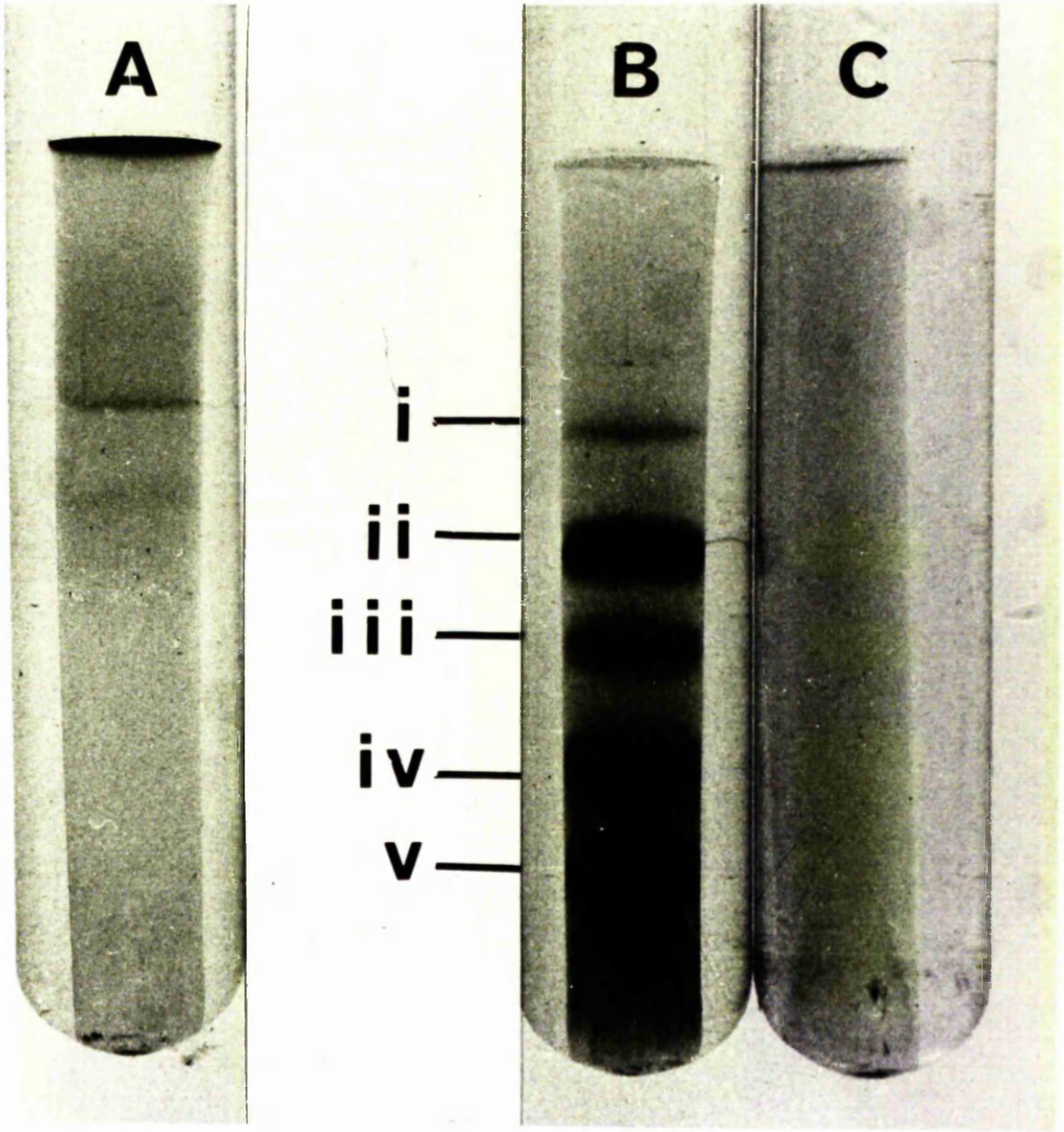
B: Standard proteins in urea-SDS system

i) hexokinase ii) bovine serum albumin

iii) ovalbumin iv) chymotrypsinogen

v) cytochrome C

C: Blank gel.


A
B
C
i
ii
iii
iv
v

(Plate III) when 6M urea and 2% (w/v) SDS were added to the sample, and 3.5% (w/v) acrylamide was employed. Unfortunately, under these conditions the relationship between the migration of standard proteins and molecular weight is non-linear. These findings indicate that α-haemolysin consists of a large molecular weight component which resists dissociation into sub-units.

2. Chemical properties.

Chemical analysis: The relative amounts of protein, total phosphorus, hexose sugars, reducing sugars and DNA were estimated in duplicate by the methods described in Materials and Methods, section H, on samples of stage II and stage III α-haemolysin. Stage III preparations were first precipitated by dialysis against 85% saturated ammonium sulphate, the precipitates were collected by centrifugation and re-dissolved in distilled water to one-tenth the original volume.

In these preparations no DNA, hexose or reducing sugars, or organic phosphorus were detected. Less than 1% inorganic phosphate was found. To test for the presence of substances other than protein, 3 ml samples of stage II α-haemolysin dissolved in TS buffer, pH 7.3, (2.93 mg/ml protein as determined by Lowry estimation) were evaporated to dryness at 95°C and then desiccated over P_2O_5 to ensure complete removal of water; 3 ml samples of the TS buffer used to dissolve the haemolysin were included as controls. Gravimetric estimation showed that Lowry-positive material contained in stage II α-haemolysin accounted for 95% of the total dry weight.

A summary of the physico-chemical properties of α-haemolysin determined in this investigation is given in Table 23.

Table 23 : Summary of the physico-chemical properties of α-haemolysin produced by E. coli strain 25238

Property		Preparation of α-Haemolysin used	
		Stage II	Stage III
Stability to;	Heat :	50% inactivation in 30 min at 37°C. 100% inactivation in 30 min at 61°C and 81°C.	NT[1]. 100% inactivation in 30 min at 56°C.
	Storage :	50% inactivation in 24 hr at 4°C No inactivation in 2 wk at -20°C and -70°C.	NT 50% inactivation in 2 wk at -20°C.
	Lyophilisation :	100% loss of activity	NT.
	Proteolytic enzymes :	100% inactivation in 20 min at room temperature with trypsin and pronase (200 µg/ml).	NT.
	Thiomersalate :	No inhibition of haemolysis	No inhibition of haemolysis
	Urea :	60% inactivation after 24 hr at 4°C in 3.5 M urea. 90% inactivation in 4 - 6 M urea.	NT.
Molecular weight:			
after Sephadex G-200 fractionation		M.W. in excess of 2×10^5	M.W. in excess of 2×10^5
by Amicon ultrafiltration		M.W. between 1×10^5 and 3×10^5	NT.
by Diffusion coefficient		NT.	M.W. between 5.0×10^5 and 6.0×10^5.
Absorption spectrum		NT.	No absorption in visible light; absorption maxima at 276 - 280 nm and 200 - 230 nm.
Chemical composition		95% protein by weight	At least 95% protein

1. NT : not tested

F. Kinetic Studies of the Haemolytic Activity of α-Haemolysin.

1. Rate measurements.

Although the substrate or receptor for α-haemolysin is not known, it was decided to perform kinetic studies using sheep erythrocytes as substrate in order to obtain information regarding the mechanism of action of α-haemolysin. Haemolysis was monitored continuously by reduction in optical density at 650 nm in the SP800 spectrophotometer which allowed rate measurements to be made on the haemolytic reaction. Stage II α-haemolysin was employed for the most part in these studies. However, at some point in each experiment, stage III (peak fraction A) was included to confirm results obtained with less pure preparations.

Figure 16 shows the time course for haemolysis using varying concentrations of α-haemolysin. The reaction was characterised, even at high concentrations of α-haemolysin, by an initial lag phase followed by a period of lysis which was linear between 20% and 80% haemolysis. By plotting the lag and rate of haemolysis against the logarithm of α-haemolysin concentration (Figure 17) it can be seen that the lag phase was inversely proportional to haemolysin concentration. The rate of haemolysis increased at a constant rate with higher concentrations of haemolysin but tailed off when less than 2×10^3 HU were added to the system.

The effect of calcium ions on the rate of haemolysis is shown in Figure 18. The rate of haemolysis was unaltered at calcium chloride concentrations between 0.1 mM to 100 mM. The length of the lag phase however, was reduced to a minimum when 1 - 10 mM calcium chloride was included in the system. In the absence of externally-supplied calcium ions slight activity did occur but the lag phase was prolonged and rate of haemolysis was slow. It was found also that magnesium (as 10 mM $MgSO_4.7H_2O$) or manganese (as 10 mM $MnCl_2$) ions would not substitute for calcium ions.

Figure 16 : The effect of α-haemolysin concentration on the kinetics of haemolysis

The α-haemolysin was pre-incubated with calcium ions for 1 min prior to addition of SRBC.

Calcium chloride concentration : 10mM (final).

Temperature 37°C.

Symbols: Final concentrations of α-haemolysin in reaction mixture.

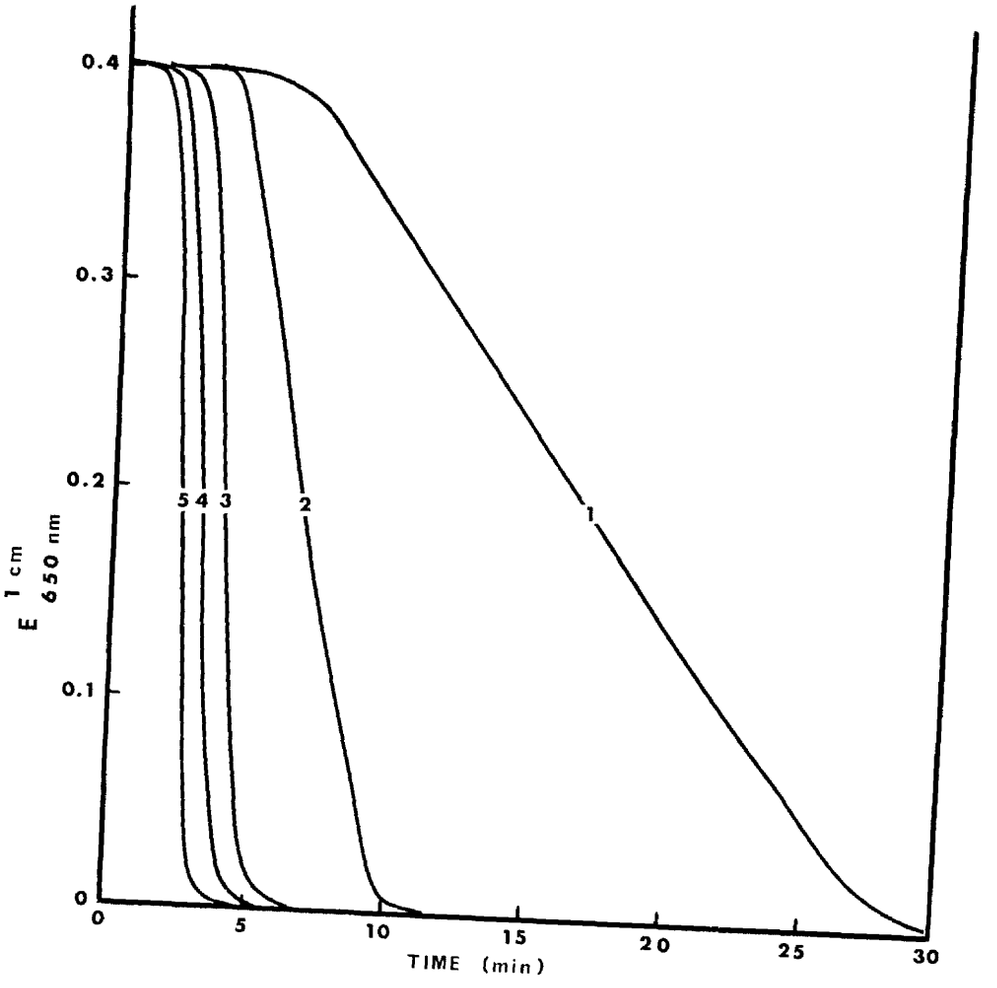
1. 1.8×10^2 HU
2. 1.8×10^3 HU
3. 1.8×10^4 HU
4. 9.0×10^4 HU
5. 1.8×10^5 HU

Figure 17 : The effect of α-haemolysin concentration on the lag phase and rate of haemolysis

Symbols:

▣ : length of the lag phase at various concentrations of α-haemolysin given in Figure 16.

O : rate of haemolysis calculated from Figure 16 at the concentrations of α-haemolysis tested.


0.4
0.3
0.2
0.1
0
$E^{1\,cm}_{650\,nm}$
5
4
3
2
1
0
5
10
15
20
25
30
TIME (min)

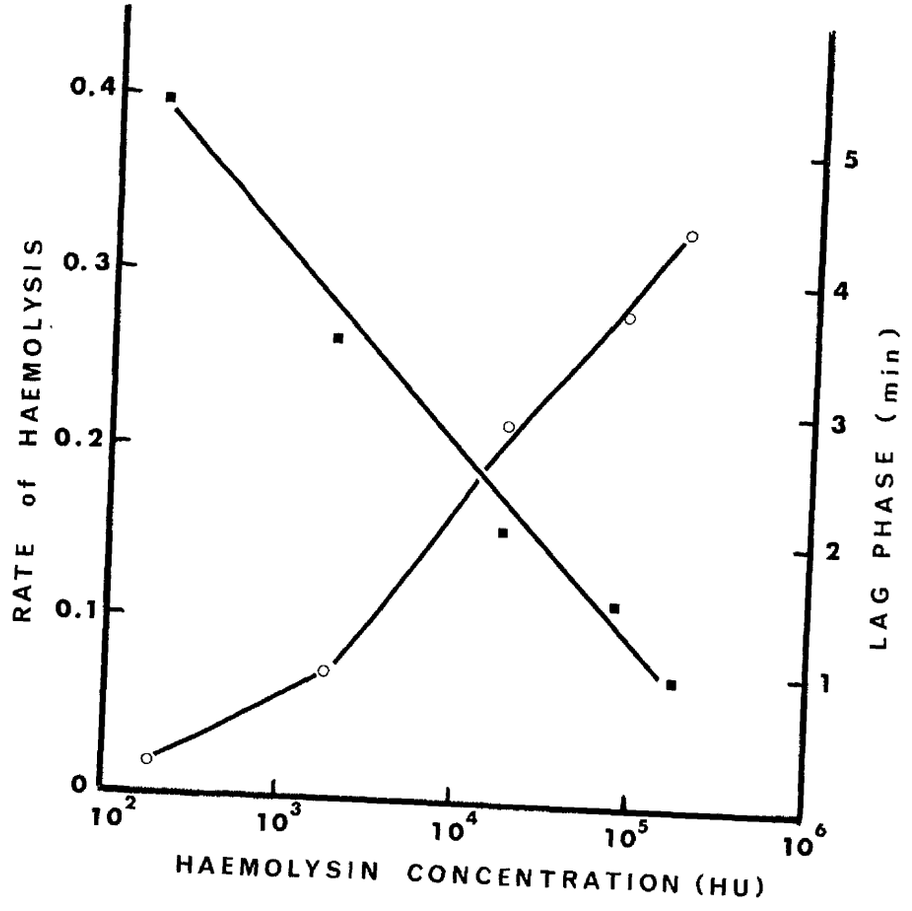

0.4
0.3
0.2
0.1
0
RATE of HAEMOLYSIS
5
4
3
2
1
LAG PHASE (min)
10^2
10^3
10^4
10^5
10^6
HAEMOLYSIN CONCENTRATION (HU)

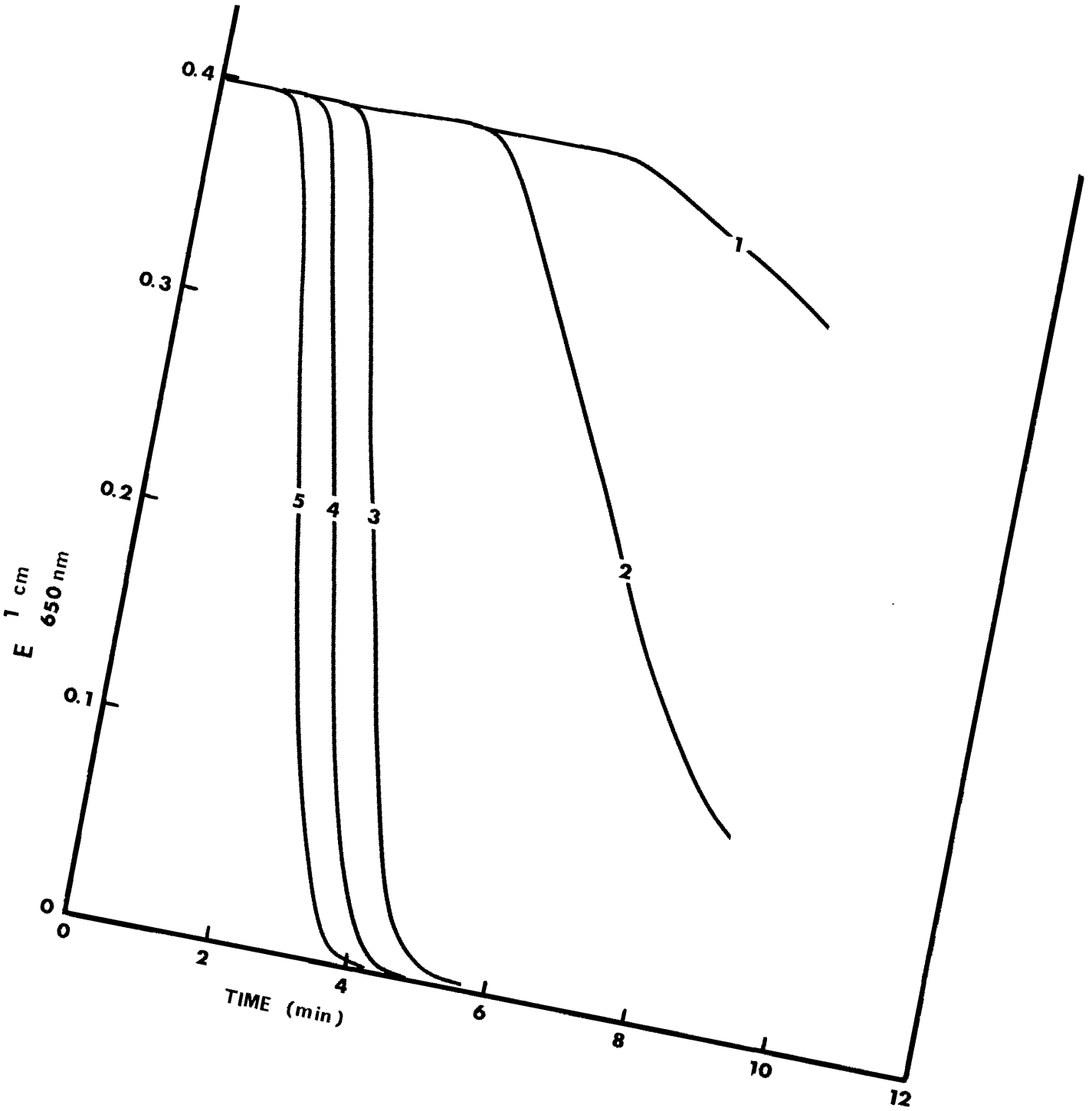
Figure 18 : The effect of calcium ion concentration on the kinetics of haemolysis

Haemolysin concentration : 90,000 HU

Temperature : 37°C

Symbols:

1. No external source of calcium ions added.
2. Final concentration of calcium chloride = 0.01mM.
3. Final concentration of calcium chloride = 100mM.
4. Final concentration of calcium chloride = 0.1mM.
5. Final concentrations of 1, 2, 5 and 10mM of calcium chloride gave an identical haemolysis curve.


0.4
0.3
0.2
0.1
0
$E^{1\,cm}_{650\,nm}$
0
2
4
6
8
10
12
TIME (min)
1
2
3
4
5

The influence of temperature and pH on haemolysis showed that the rate of haemolysis increased as both variables increased (Figure 19). Maximum rates were approached at pH 8.8 to 9.0 and a temperature of 43°C. The lag phase was not affected, except in experiments performed at 25°C and below pH 7.0 when increased lag and decreased rates of haemolysis were observed.

The studies mentioned above showed that a lag phase was required in all cases before haemolysis could occur. It was therefore decided to investigate this part of the haemolytic reaction in more detail. In initial experiments α-haemolysin, in buffer containing calcium ions, was incubated for 1 min at 37°C to bring the buffer and haemolysin to the temperature of the system before adding SRBC. To obtain further information regarding the role of calcium ions in the haemolytic reaction, α-haemolysin was pre-incubated with 10 mM calcium chloride for various periods of time before addition of SRBC. Figure 20 shows that, at the concentration of haemolysin used, pre-incubation with calcium ions to a maximum of 5 min caused a progressive reduction in the length of the lag phase; the rate of haemolysis was not affected. Similar reaction curves were seen when haemolysin was pre-incubated with SRBC before addition of calcium chloride (Figure 21).

A series of kinetic experiments were performed to determine if calcium was required throughout the course of haemolysis. A lower concentration of α-haemolysin (3000 HU) and less calcium chloride (2 mM) were used in order to prolong the lag period, thus allowing several procedures, as outlined in the legend to Figure 22, to be performed.

From Figure 22, the following observations were made:

a) EDTA by itself did not cause lysis of SRBC (1).

b) EDTA inhibited haemolysis when pre-incubated along with α-haemolysin

Figure 19 : The effect of pH and temperature on rate of haemolysis

Haemolysin concentration = 9×10^4 HU

Calcium chloride concentration = 10 mM

Symbols:

■ : rate of haemolysis at various pH values using 0.01M Tris-HCl diluent. Temperature of incubation; 37°C.

○ : rate of haemolysis at various incubation temperatures using Veronal diluent, pH 7.3.

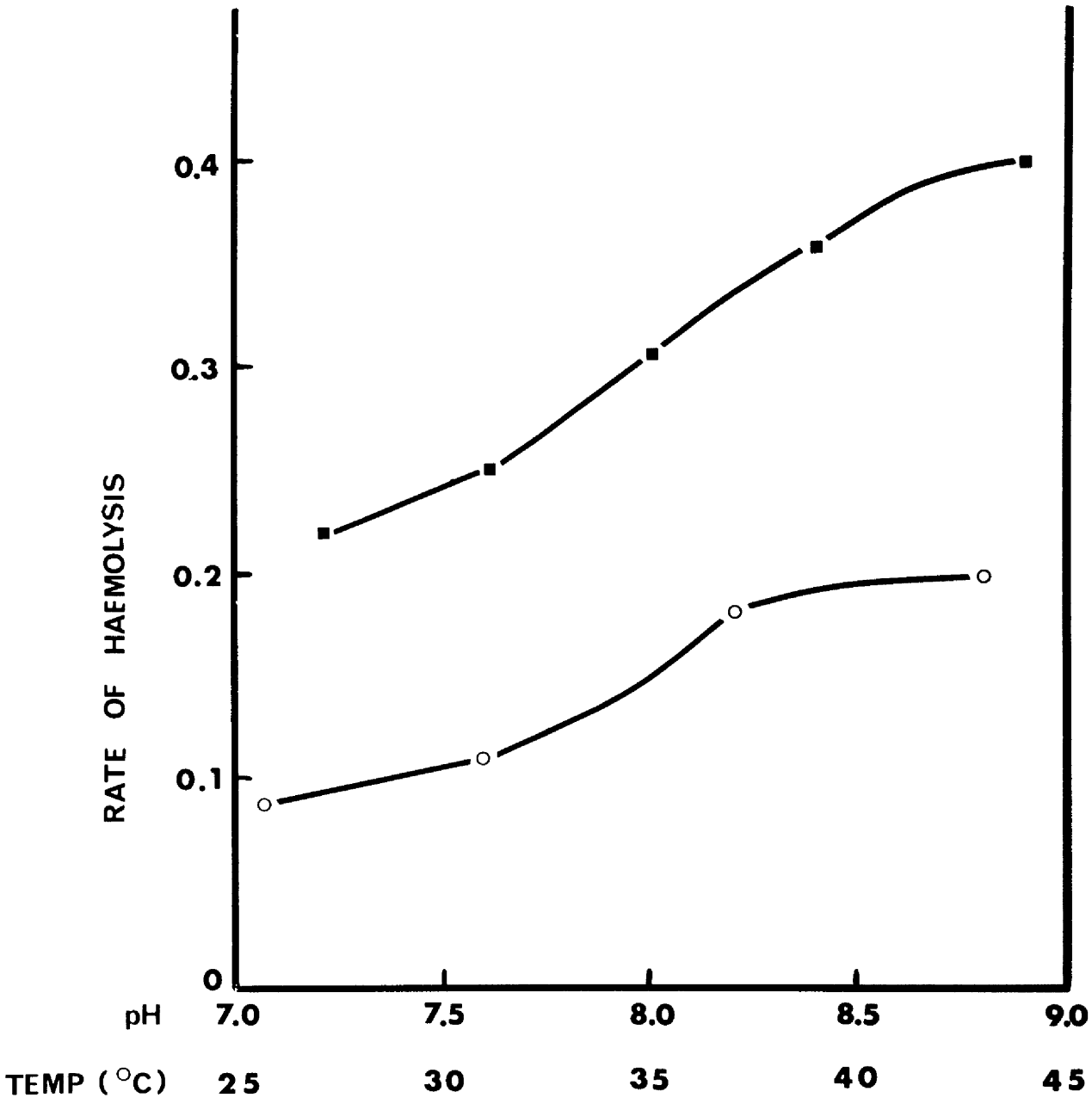

RATE OF HAEMOLYSIS
0.4
0.3
0.2
0.1
0
pH
7.0
7.5
8.0
8.5
9.0
TEMP (°C)
25
30
35
40
45

Figure 20 : The effect of incubating haemolysin and calcium ions prior to initiating haemolysis by adding sheep red blood cells.

Haemolysin concentration = 90,000 HU
Calcium chloride concentration (final) = 10 mM
Temperature = 37°C

Symbols:

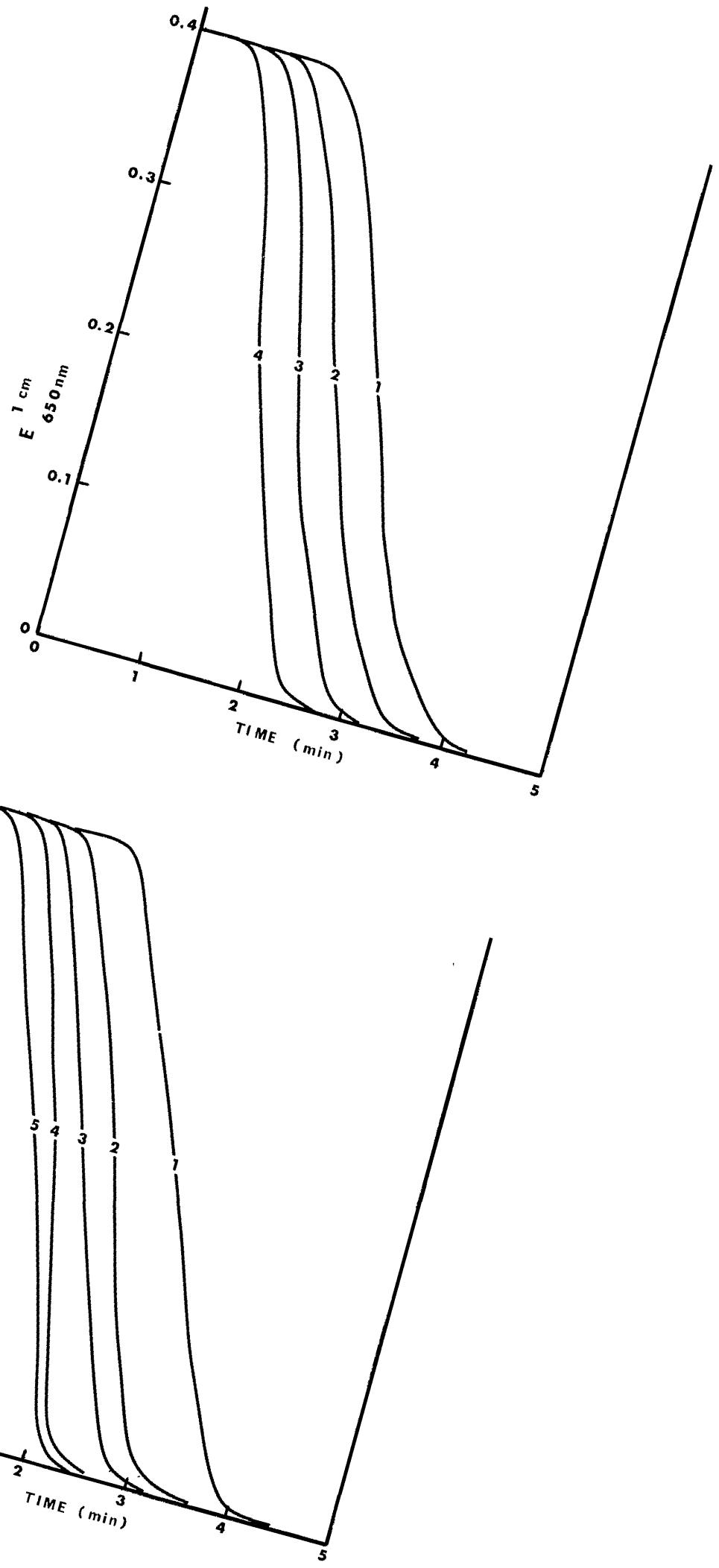
1. No pre-incubation of α-haemolysin with calcium chloride before adding SRBC.
2. α-haemolysin pre-incubated for 1 min with calcium chloride.
3. α-haemolysin pre-incubated for 2 min with calcium chloride.
4. α-haemolysin pre-incubated for 5 - 20 min with calcium chloride (identical reaction curves).

Figure 21 : The effect of incubating haemolysin and sheep red blood cells prior to initiating haemolysis by adding calcium chloride to a concentration of 10 mM

Haemolysin concentration = 90,000 HU
Temperature = 37°C

Symbols:

1. SRBC and calcium ions added at the same time.
2. α-haemolysin pre-incubated for 1 min with SRBC.
3. α-haemolysin pre-incubated for 2 min with SRBC.
4. α-haemolysin pre-incubated for 5 min with SRBC.
5. α-haemolysin pre-incubated for 8 min with SRBC.


0.4
0.3
0.2
0.1
0
E 1cm 650nm
0
1
2
3
4
5
TIME (min)
4
3
2
1
0.4
0.3
0.2
0.1
0
E 1cm 650nm
0
1
2
3
4
5
TIME (min)
5
4
3
2
1

and calcium ions (2), but did not affect the haemolytic reaction after α-haemolysin had been activated by calcium ions (6). The apparent increase in the lag phase observed in (6) as compared to the control haemolytic reaction (7) was due to a cooling period (2 min at 4°C) carried out for reaction (6). This was done in order to delay haemolysis and perhaps exaggerate any inhibitory effect of EDTA.

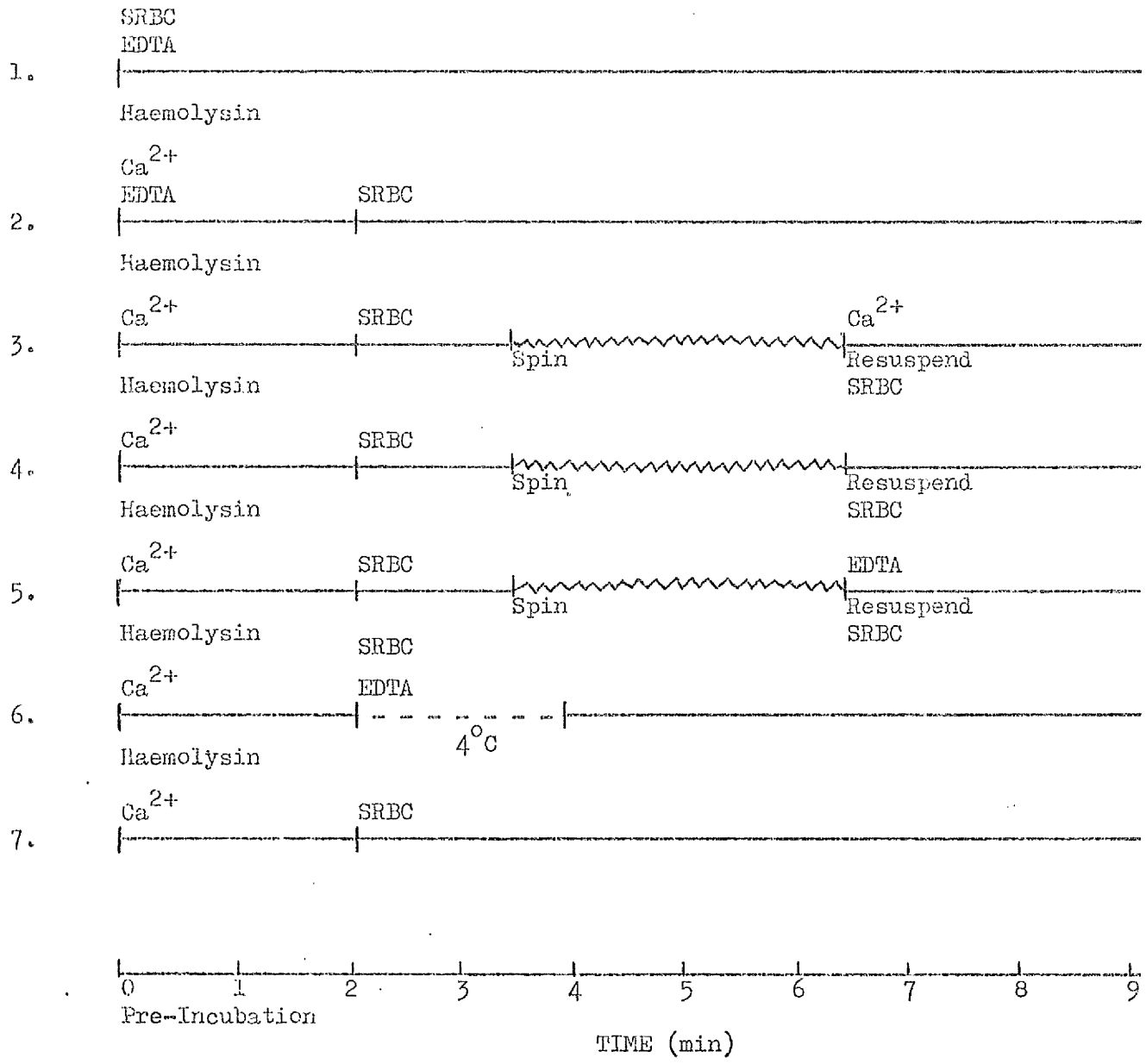
c) The apparent inhibition of haemolysis seen in reaction curves (3), (4) and (5) was due to removal of residual α-haemolysin and calcium ions left in the supernatant following centrifugation of the reaction mixture (1.5 min after adding SRBC). This procedure was performed to see whether calcium ions were required for haemolysis after at least a portion of the α-haemolysin contained in the original reaction mixture had interacted with SRBC. The portion of α-haemolysin, contained in the pellet of SRBC after centrifugation, neither required a further source of calcium ions to lyse those red cells with which it had interacted (3), nor was inhibited if calcium ions were not added back to the reaction mixture (4). Also, inhibition of haemolysis was not observed if EDTA was added when the red cells were resuspended (5). This indicated that calcium ions were not required after interaction between α-haemolysin and erythrocytes had occurred.

2. Electron microscopy.

Samples of stage III α-haemolysin were dialysed against distilled water to remove salts which might affect resolution in the electron microscope. Thus prepared, 1 mM calcium chloride was added to one sample; the other was left untreated. After 10 min at 37°C, aliquots of treated and untreated samples were placed on carbon-coated copper grids and dried *in situ*. Negative-staining was performed with 2% (w/v) aqueous ammonium molybdate and the samples were examined in a Phillips EM300 electron microscope operating at 60 Kv.

Figure 22 : The effect of EDTA on activated α-haemolysin

Explanation to figure :



Additions to spectrophotometer cells are indicated above the horizontal lines; manipulations (e.g. centrifugation followed by resuspension of SRBC or alteration of the temperature of incubation at $4^{\circ}C$) are indicated below the lines. Horizontal lines where continuous indicate incubation at $37^{\circ}C$.

α-haemolysin concentration = 3000 HU.

Calcium chloride concentration = 2 mM.

EDTA concentration = 5 mM.

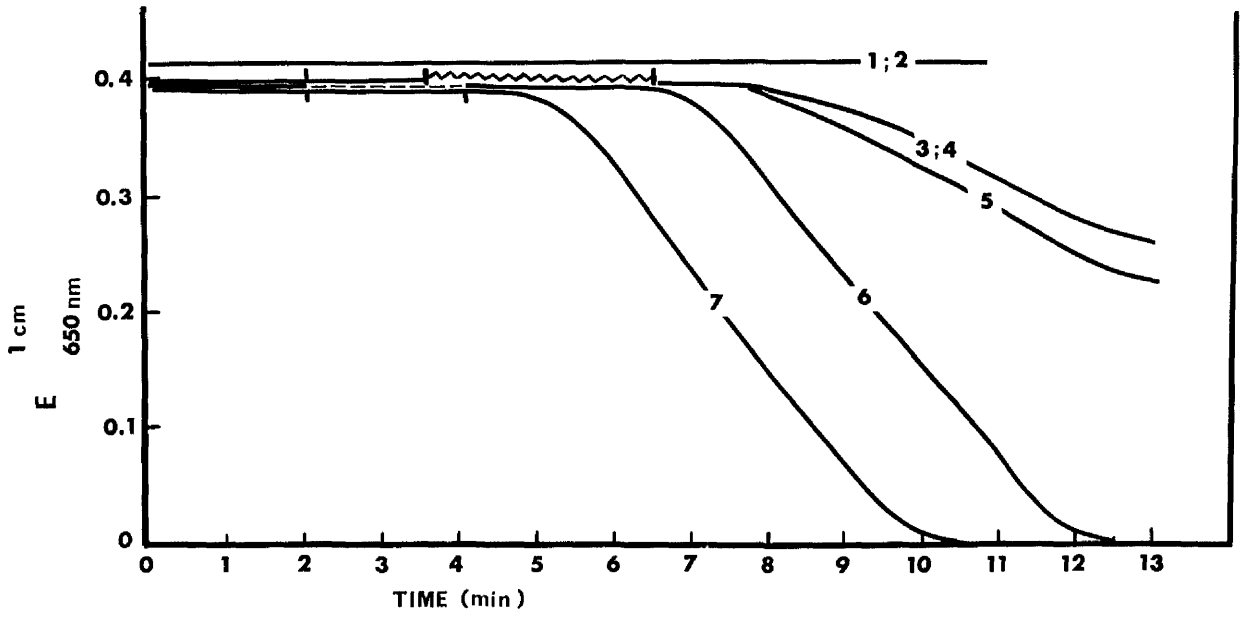

1;2
3;4
5
6
7
0.4
0.3
0.2
0.1
0
E 1 cm 650 nm
0 1 2 3 4 5 6 7 8 9 10 11 12 13
TIME (min)

Plate IV shows untreated α-haemolysin which consisted mainly of amorphous strand-like structures. By contrast, α-haemolysin treated with calcium orientated into regular structures with a spherical appearance (Plate V). Some of these latter forms were seen in untreated preparations.

Random measurement of the diameters of 100 of the structures seen in Plate V gave a normal distribution curve with a maximum at 15 nm. Direct conversion of this size to molecular weight was made according to the formula:

$$M = \frac{N}{\bar{V}} \times V,$$ where N = Avogadro's number,

$\bar{V}$ = partial specific volume (assume a value of 0.73), and

V = calculated volume of the sphere (see Green, 1969).

If the structures seen in Plate V were assumed to be collapsed spheres with thickness 0 nm a calculated molecular weight of 1.5×10^5 was obtained. Assuming an uncollapsed sphere, a calculated molecular weight of 5.8×10^5 was obtained. Both assumptions were considered because of the two-dimensional nature of objects seen in the electron microscope.

G. Biological Properties.

1. Erythrocyte sensitivity.

The sensitivity to α-haemolysin of various erythrocyte species was investigated using a spectrophotometric assay for haemoglobin release. The erythrocytes were collected in 3.8% (w/v) sodium citrate and were used within 3 days of collection; stage II α-haemolysin was employed for these studies.

From Table 24, it can be seen that, compared to sheep erythrocytes, rabbit erythrocytes were equally sensitive to α-haemolysin. By contrast, erythrocytes obtained from 3 species of fish were almost totally resistant.

Plate IV. Electron micrograph of stage III α-haemolysin in the absence of calcium ions.

A sample of stage III α-haemolysin (peak fraction A) was dialysed against distilled water for 24 hr to remove buffer salts. An aliquot was incubated for 10 min at 37°C; it was then placed on a carbon-coated copper grid and dried *in situ*. Negative-staining was performed with 2% aqueous ammonium molybdate.

Note the elongated structures (arrow) with some spherical particles (circle).

Magnification: X 72,600

These structures are seen also at a higher magnification (see insert : Magnification X 195,000).

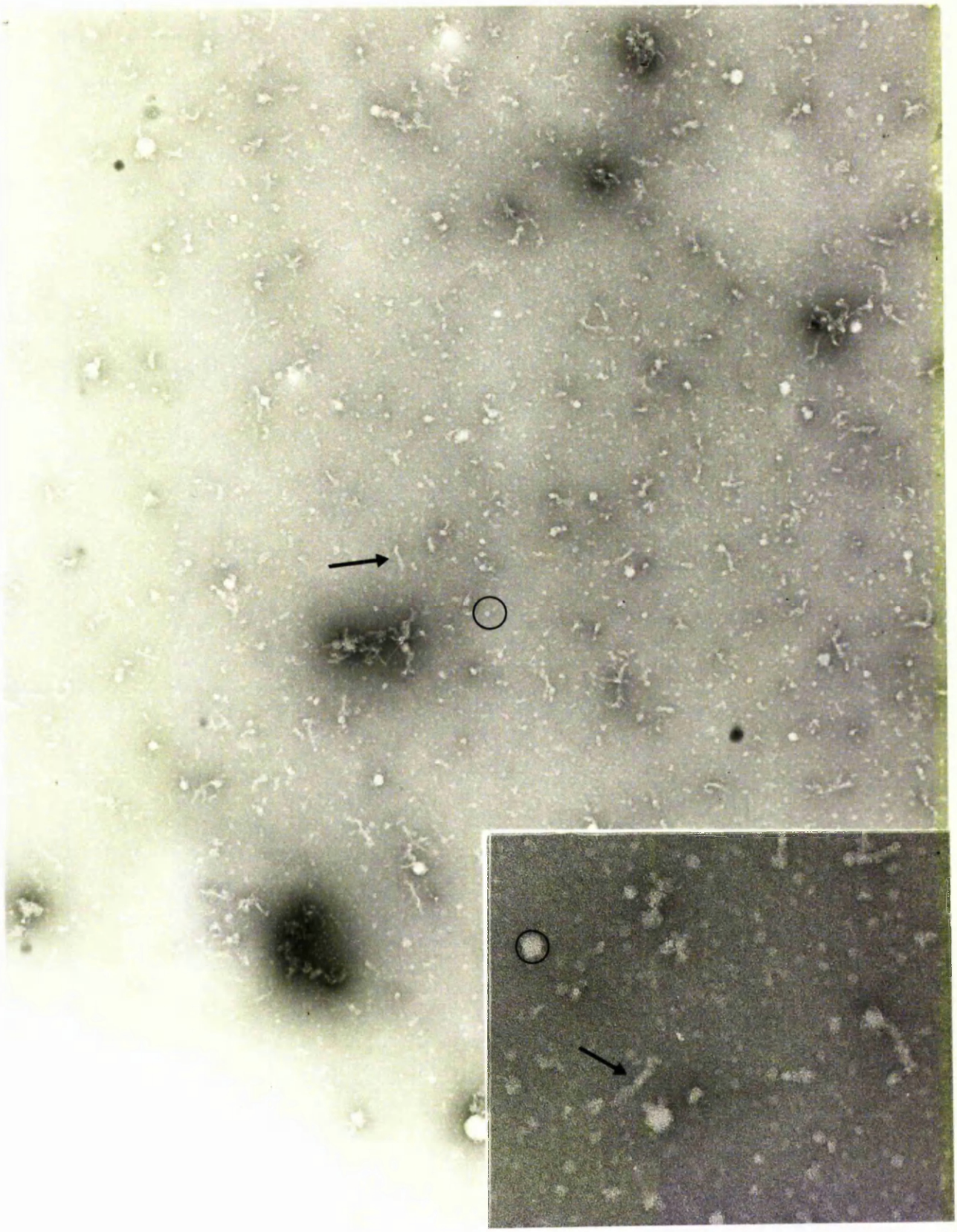


Plate V. Electron micrograph of stage III α-haemolysin after activation by calcium ions.

A sample of stage III α-haemolysin (peak fraction A) was dialysed against distilled water for 24 hr to remove buffer salts. To an aliquot of haemolysin was added 1 mM (w/v) calcium chloride. After incubation for 10 min at 37°C the sample was placed on a carbon-coated copper grid and dried *in situ*. Negative-staining was performed with 2% aqueous ammonium molybdate.

Note the large number of uniform spherical structures (circle). A few elongated structures (arrow) can be seen.

Magnification X 72,600.

These structures are evident also at a higher magnification (see insert, magnification X 195,000).

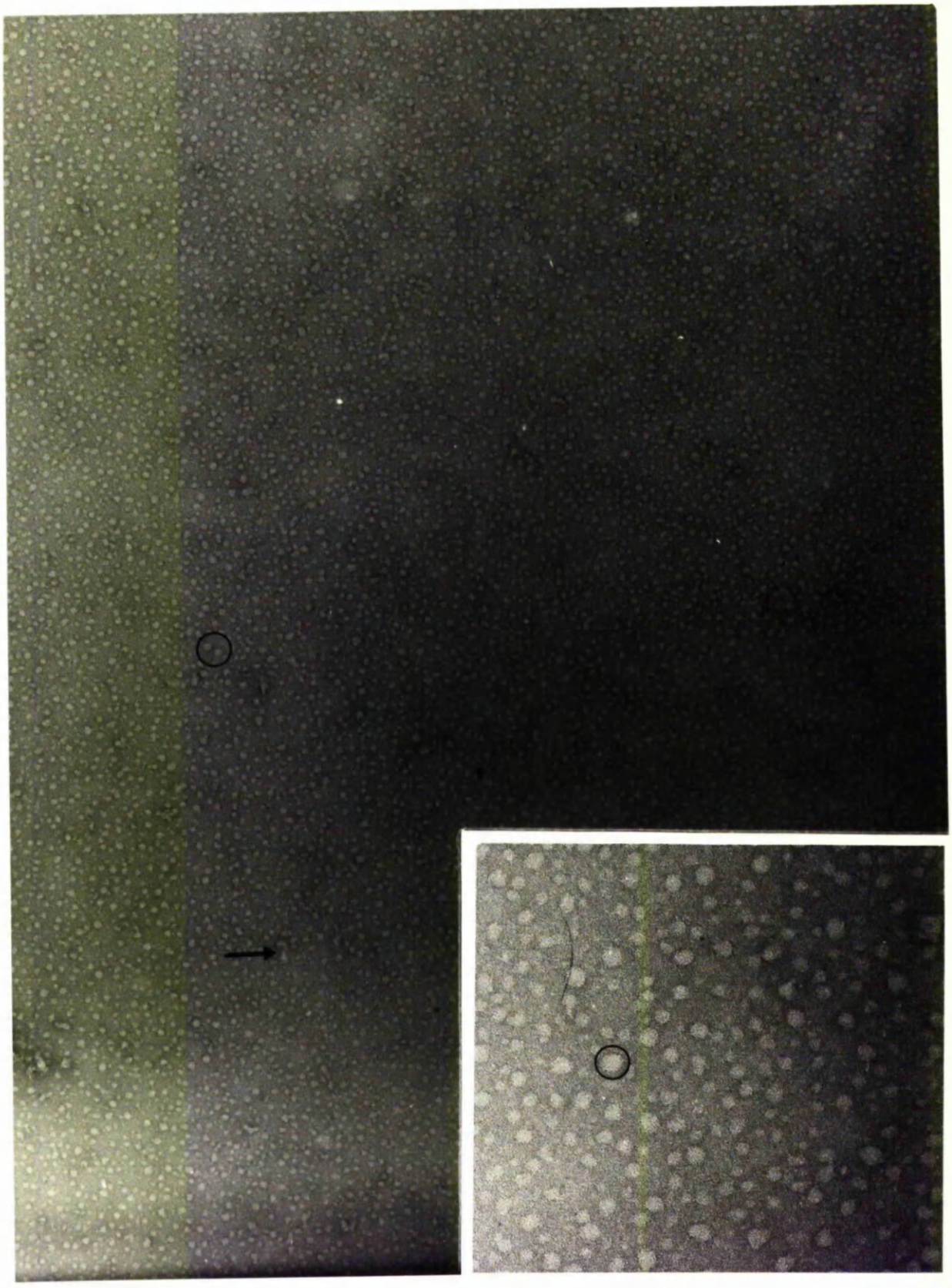


Table 24 : Haemolytic spectrum of *E. coli* α-haemolysin

Erythrocyte Species	% Relative Activity[1.]
Sheep	100
Rabbit	100
Baboon	83.3
Cat	75
Dog	66
Cow	62.5
Horse	59
Rat	50
Human (AB)	27
Mouse	20.8
Fish: Cod	0.08
Haddock	0.08
Saithe	0.04

1. Sheep erythrocytes as 100%

Among mammalian erythrocyte species, most of those tested showed a relatively high sensitivity to α-haemolysin. It was interesting to note that baboon erythrocytes were very sensitive when compared to human group AB erythrocytes.

2. Effect of α-haemolysin on phospholipid emulsions.

Attempts were made to find a single substrate for α-haemolysin. A sample of purified stage III α-haemolysin (fraction A) was incubated for 24 hr at 37°C with emulsified suspensions of L-α-lecithin and sphingomyelin. Purified phospholipase C (20 µl) from Clostridium perfringens (see Smyth, 1972) was included as a positive control. After incubation, treated and untreated preparations were analysed for release of water-soluble phosphorus. Also, aliquots of chloroform extracts of these samples were chromatographed by TLC.

No release of water-soluble phosphorus, as estimated by the "½ Allen" method (see page 62) was found after treatment of either lecithin or sphingomyelin with purified α-haemolysin. The positive phospholipase control caused the release of 10 µg phosphorus from lecithin and 13 µgphosphorus from sphingomyelin. Also, neither displacement of spots nor appearance of additional spots, indicating hydrolysis of the phospholipid, were found when α-haemolysin treated and untreated phospholipids were compared by TLC. By contrast TLC of phospholipids treated with the positive phospholipase control revealed that hydrolysis of both substrates had occurred.

3. Neutralisation of haemolytic activity.

Initial attempts were made to produce in rabbits an antiserum to stage I α-haemolysin according to the method of Smith (1963). As measured by tube test, only low levels of anti-α-haemolysin (1200 anti-α-units/ml) were formed using Smith's method of antiserum production. Moreover, when this antiserum was incorporated into wells cut in erythrocyte agar overlay medium

and tested against haemolytic E. coli strains isolated from inpatients of the hospital survey, wide variations occurred in inhibition of haemolytic activity. Zones of haemolysis produced by some strains were inhibited to within 6 - 8 mm of the antiserum well; haemolytic zones produced by other strains were not inhibited.

When more purified stage II α-haemolysin became available, antiserum produced in rabbits by the method of Gallop et al., (1966) had 70 - 80 times greater potency than anti-stage I antiserum as monitored by tube test (Table 25). Also, of the haemolytic strains isolated from inpatients, 32 of these were re-tested using the potent antiserum. Zones of haemolysis produced by some of these strains had not been inhibited by anti-stage I antiserum. From Plate VI it can be seen that the potent anti-stage II antiserum neutralised zones of haemolysis produced by the 6 strains examined for production of α-haemolysin in NBG medium. Similar neutralisation was obtained with the other 26 strains. Thus, it appears obvious that the method of Smith gave a low avidity antiserum.

Anti-α-haemolysin did not neutralise cell-associated, β-haemolysin produced by strain 25238 in CDM. However, suspensions of antiserum and β-haemolytic cells became cloudy during incubation, and it is probable that substances contained in the antiserum preparations were utilised as growth factors by viable E. coli cells.

4. Tissue culture toxicity.

Studies of the effect of stage II α-haemolysin on toxicity of HeLa cells showed that very high concentrations of α-haemolysin were required to kill a monolayer of these cells (Figure 23). Heat inactivated α-haemolysin was non-toxic even at the highest concentration used. In addition, varying the calcium chloride concentration between 2 and 20 mM (w/v) did not affect the response of HeLa cells to α-haemolysin. BHK cells were totally resistant to α-haemolysin.

Table 25 : Neutralisation of α-haemolytic activity by tube tests.

Antigen	Antiserum Preparation[1].	Total Anti α-Haemolytic Units
Stage II α-haemolysin	Pre-immunisation	160
	Test bleed (19 days)	2,000
	Test bleed (50 days)	80,000

1. Antiserum produced in rabbits according to the method of Gallop et al., (1966).

Plate Vi. Neutralisation of the haemolytic activity of *E. coli* strains on agar plates.

Symbols:

A : strain 25238 = urine isolate : O19:B7.

B : strain 4748 = urine isolate : O6.

C : strain 122 = throat isolate : O86:B7.

D : strain 189 = throat isolate : O128:B12.

E : strain 3829 = wound isolate : O6.

F : strain 15370 = umbilical cord isolate : O18:B21.

A_S: Antiserum to stage II α-haemolysin.

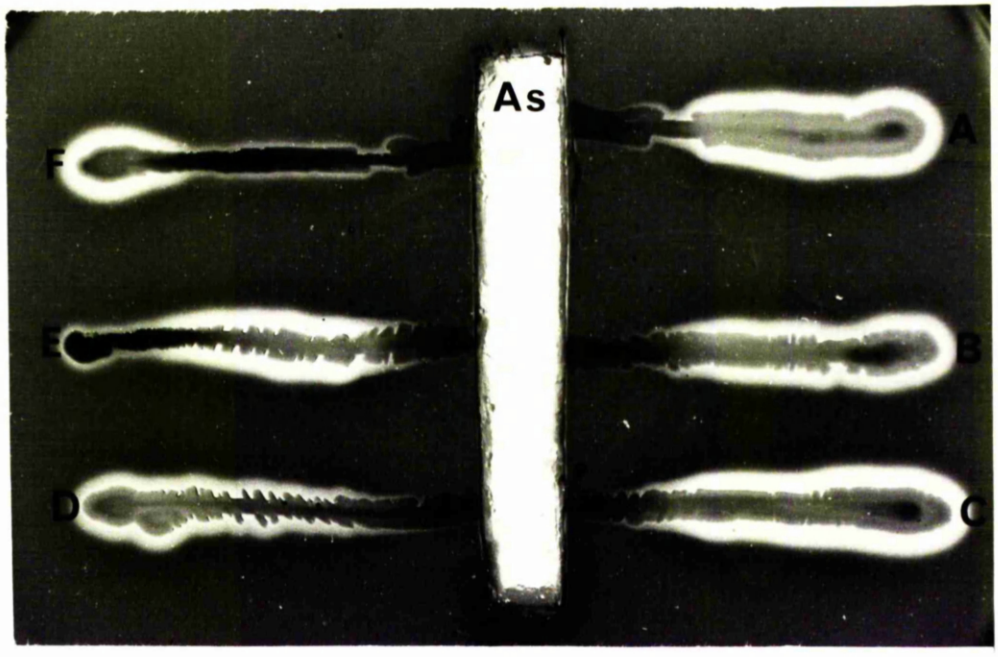

As
A
B
C
D
F

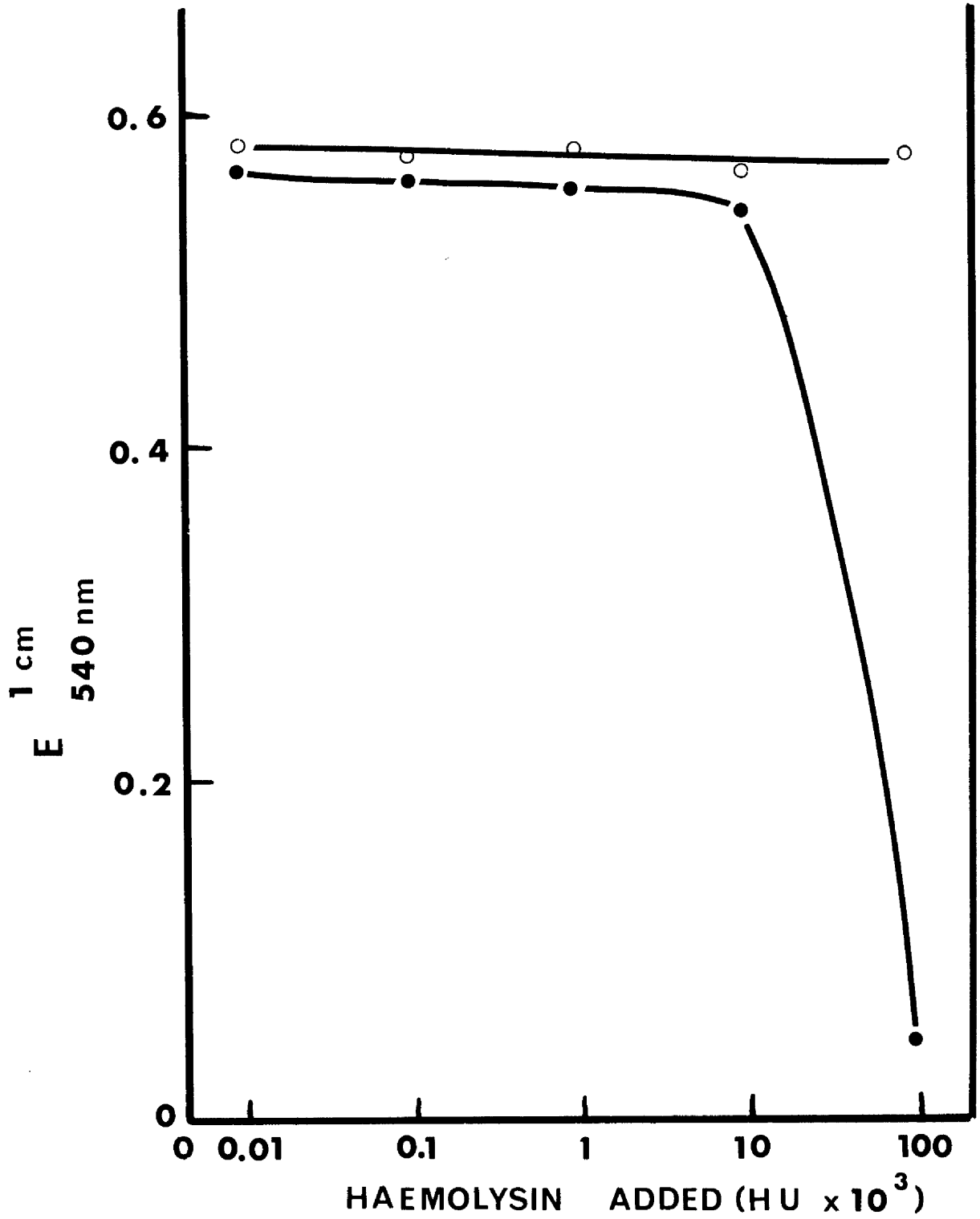
Figure 23 : The effect of α-haemolysin on Hela tissue culture cells; neutral red assay

Symbols:

⊗ : $E^{1cm}_{540\ nm}$ of neutral red extract of Hela cells treated with α-haemolysin.

○ : $E^{1\ cm}_{540\ nm}$ of neutral red extract of untreated Hela cells.

Each point represents the amount of neutral red dye taken up by viable cells in 4 tubes containing monolayers of Hela cells.


0.6
0.4
0.2
0
E 1 cm 540 nm
0
0.01
0.1
1
10
100
HAEMOLYSIN ADDED (H U x 10 3)

5. Effects of α-haemolysin in mice and rabbits.

Of 16 mice injected intravenously with stage II α-haemolysin or heat-inactivated haemolysin, none showed any signs of ill-health and no deaths were recorded after 48 hr. One rabbit injected intravenously with 3 mg of stage II α-haemolysin remained well.

After intradermal injection of stage II haemolysin into the shaved backs of rabbits, hard swellings appeared at the injection site. The diameter of the swellings, proportional to the amount of haemolysin injected, increased in magnitude until 18 - 20 hr after injection and decreased during the following 48 hr (Table 26). This response was observed also when 25 μg of stage III haemolysin (fraction A) was injected, but not when fraction B was administered. The highest concentration of stage II haemolysin used, when heated for 1 hr at 56°C, did not cause swelling; neither did sterile saline, TSG buffer nor sterile NBG medium treated in the same way as for the preparation of stage II α-haemolysin.

When 40 mg/kg of a saline solution of Evans Blue dye was injected intravenously 18 hr after intradermal injections, according to the method described by Moon and Whip (1971) for determination of vascular permeability factors contained in E. coli enterotoxin preparations, intense blueing resulted both in swollen and unswollen areas of the rabbits.

6. Ligated intestinal loop test in rabbits.

The response to α-haemolysin of ligated intestinal loops prepared in rabbits has not been investigated before. It was therefore of interest to perform at least preliminary experiments in this test system. The fact that only a few experiments could be done was due to Home Office regulations relating to the issuing of certificate B for surgical procedures. It was therefore necessary to perform these experiments with the assistance of

Table 26 : Intradermal injection of rabbit skin with α-haemolysin

Preparation	Protein Injected (μg)	Average Diameter of Induration (mm)*
Stage II Haemolysin	350	18.5
	35	15.0
	3.5	10.5
	0.35	0
Stage III, Peak A	25	13.1
Stage III, Peak B	15	0

* An average of 4 measurements recorded for each dose

veterinary surgeons and operating theatre nurses who kindly took time out from their regular duties. However, it should be stressed that strict aseptic precautions were possible because of the proper operating facilities available at the University of Glasgow Veterinary School.

Initial experiments conducted on three rabbits confirmed the observations of Smith and Halls (1968b) and others that the first 30 to 40 cm of the anterior portion of the small intestine was unsuitable. In these rabbits fluid accumulation was observed irrespective of whether the loops were injected or not and all 3 rabbits died within 10 - 12 hr after surgery.

Observations using a further 8 rabbits in which the first ligature was made at least 45 cm posterior to the pylorus are recorded in Table 27. Though it must be recognised that these experiments represent only preliminary findings, four points are worth noting:

1. None of the preparations used caused dilatation and fluid accumulation of injected loops.

2. The macroscopic appearance of loops injected with sterile saline, heated stage II α-haemolysin or a non-haemolytic strain of E. coli, serotype 055:K59(B5)H6, an enteropathogenic serotype isolated originally by Giles et al., (1949) was similar to uninjected control loops which separated the test loops.

3. Loops injected with strain 25238 or stage II α-haemolysin had a haemorrhagic appearance when the small bowel was excised at autopsy: one rabbit, in which 3 test loops were prepared and injected only with stage II haemolysin died 8 hr after surgery although no outward evidence of agony was observed until a few minutes before death.

4. At autopsy, the haemolytic strain (serotype 019:B7:H?) was isolated from heart blood in those rabbits in which it had been injected; the

Table 27 : Summary of ligated intestinal loop tests in rabbits

Preparation	No. of Rabbits	No. of Test Loops	Macroscopic Appearance of Injected Loops	Bacteriological Findings*			
				Loop Contents		Blood Culture	
				+	−	+	−
E. coli strain 25238	3	9	Dark, reddish colour; haemorrhagic in appearance.	6/6		3/3	
E. coli strain Aberdeen β 055:K59(B5):H6	2	6	Similar to uninjected control loops.	4/4			2/2
Stage II α-haemolysin	1	3	Dark, reddish colour; haemorrhagic in appearance.		2/2		1/1
Stage II α-haemolysin heated for 1 hr at 60°C.	1	3	Similar to uninjected control loops.		2/2		1/1
Sterile 0.85% (w/v) saline	1	3	Similar to uninjected control loops.		2/2		1/1
None (control loops)	8	32	Normal appearance.		16/16	NT	NT

*
(+) Lactose-positive bacteria isolated
(−) No lactose-positive bacteria isolated
NT Some control loops were in rabbits from which strain 25238 was isolated from the heart blood.

non-haemolytic strain, serotype 055:K59(B5):H6 was isolated only from ligated loops into which it had been injected.

DISCUSSION

DISCUSSION

A. General Remarks.

The reasons for studying the haemolytic activity of E. coli have been mentioned previously but the main points should be summarised here. One of the aims of the present investigation was to see what role, if any, E. coli haemolysin plays in pathogenicity, especially in enteric infection. However, in order to perform meaningful experimental studies of the biological properties of the haemolytic principle it was essential a), to obtain large amounts of high titre haemolysin in culture filtrates and b), to achieve a satisfactory degree of purification. It soon became obvious that the major problem experienced by the few workers who have attempted to characterise E. coli haemolysin was the inability to satisfy these criteria.

In this laboratory, high titre E. coli haemolysin has been produced and purified to a considerably greater degree than previously reported. Also, progress has been made in the search to determine the mechanism of action of E. coli haemolysin at the molecular level and the significance of this bacterial product in pathogenicity. The broader implications of extracellular products of gram-negative bacteria are also discussed.

B. Growth and Haemolysin Production.

1. Synthesis of β-haemolysin in chemically-defined medium.

In contrast to gram-positive bacteria, few gram-negative bacteria liberate extracellular products into the culture medium during logarithmic growth (Lampen, 1965). Certainly, E. coli is not known to liberate significant amounts of extracellular protein (Pollock and Richmond, 1962) and, it has been suggested that, because of the complex nature and high lipid content of cell envelopes of gram-negative bacteria, a physical barrier may prevent secretion

of extracellular products from these organisms (Raynaud and Aleuf, 1970).

When haemolytic E. coli strains were grown in CDM, only cell-associated, β-haemolysin was produced; extracellular, α-haemolysin was not found. As observed in Table 15, it is probable that the haemolytic activity of supernatant fluids from CDM cultures was due to the activity of viable organisms. Although E. coli alkaline phosphatase has been found in isolated membrane fractions (Bishop, Roche and Nisman, 1964) it is unlikely that supernatant haemolytic activity of CDM-grown cultures was due to haemolysin contained in cell fragments which would not pass through membrane filters. Supernatant activity did not increase with age of the culture, as would be expected if autolysis was responsible for the appearance of supernatant haemolysin.

The almost immediate appearance of β-haemolysin after growth of the test strain in alternate enrichment and growth-limiting conditions invites discussion (see Figure 5). One explanation for this phenomenon is rapid induction of β-haemolysin synthesis following starvation conditions. It has been shown by Coffman, Norris and Koch (1971) and others that induction of β-galactosidase synthesis proceeded within 4 - 6 min after medium enrichment. Koch (1971) has suggested that, even under growth-limiting conditions, cells make ribosomal RNA, transfer RNA and ribosomal protein, but that these are not used efficiently. In the case of β-haemolysin, rapid appearance of relatively large amounts in stage C cells might be due to medium enrichment resulting in accelerated protein synthesis. Only lactose and sucrose (both disaccharides), added at the end of stage B, gave poor titres in 0 hr, stage C cells. Other sugars tested gave approximately the same initial amounts of β-haemolysin in stage C.

Another possibility, as suggested by the appearance in most experiments, of equivalent levels of β-haemolysin in 3 - 3.5 hr stage A cells

and 0 hr stage C cells, is that β-activity formed during stage A was masked in some way during starvation conditions (stage B). This condition implies a structural or chemical alteration in the cell envelope due to carbon and energy limitation. Increased capsular polysaccharide is unlikely; accumulation of lipid may occur through intermediary metabolites with a reverse effect stimulated by addition of glucose (Dagley and Johnson, 1953). A useful experimental approach would be to radioactively label stage A cultures. By comparing the β-haemolytic activity with the amount of label in whole cells, at the end of stage A, during stage B and at the start of stage C, estimation of the relative amounts of newly synthesised and pre-formed haemolysin should be possible.

2. Production of extracellular, α-haemolysin.

A notable feature of α-haemolysin was its failure to be produced in chemically defined medium (CDM). In fact, only 3 of the complex media tested supported production of α-haemolysin by strain 25238 (see Table 18). Of these, best results were obtained in NBG medium which had three major advantages.

1. In contrast to the laborious and empirical procedures required for preparation of meat-extract infusion media used by Smith (1963) and others, Oxoid Nutrient Broth No. 2 required only autoclaving and addition of filter-sterilised glucose.

2. Large amounts of high titre α-haemolysin were produced consistently. This is probably because the constituents of NBG medium did not vary appreciably in different batches.

3. The incubation time necessary for maximum production of α-haemolysin in NBG medium was short (2 hr) which allowed growth and harvesting procedures to be performed easily during a day.

These developments, by making available large amounts of E. coli haemolysin, were valuable for studies of the purification and characterisation of this agent.

Synthesis and release of α-haemolysin: Why was α-haemolysin elaborated only in certain complex media and not in CDM? In a consideration of possible reasons for this phenomenon it is desirable at this point to discuss the relationship between α- and β-haemolysin. Several lines of inquiry presented in this thesis suggest that α-haemolysin is a released form of cell-associated, β-haemolysin.

The finding that large components (M.W. $> 1 \times 10^5$) contained in nutrient broth, enhanced titres of α-haemolysin without affecting growth (see Figure 8) supports the suggestion of Inukai and Kodama (1965) that large molecular weight proteins contained in certain culture media are related to release of haemolysin from cells rather than to its synthesis. It is noteworthy in this regard that peptides with chain lengths greater than 5 - 6 amino acid residues are not generally utilised by E. coli (Payne and Gilvarg, 1968), an observation which indicates that peptidase activity is not a prominent feature of E. coli cells.

The extraction studies of Ginsburg et al., (1965) on the cell-bound form of streptolysin S provided a simple means of testing for the release of haemolysin from viable E. coli cells. It is interesting that the only 'carrier' compound which allowed the appearance of E. coli haemolysin in culture filtrates was a protein (BSA). Although synthesis of a new haemolysin in this situation cannot be excluded, it seems likely that during growth in CDM, the haemolysin has a high affinity for components of the outer layers of the cell. Thus, release of only small amounts of haemolysin would indicate that BSA is a poor carrier for α-haemolysin or, put another way, BSA does not effectively reduce the affinity of haemolysin for envelope components such that it becomes the preferred molecule for binding of haemolysin. The observations made with 6 different strains in NBG medium (see page 86) are consistent with this interpretation. It seems likely that in some strains, e.g. 25238, haemolysin

is less firmly bound to the cell surface than in other strains, e.g. 15370. Notably, the haemolytic activity of all 6 strains was inhibited by antibody to the extracellular haemolysin (see Plate VI).

Evidence that α- and β-haemolysin are two forms of the same substance comes also from similarities in their physical properties. Both forms require calcium ions for activity. Both are inactivated by heat and by incubation with trypsin; β-haemolytic activity which re-appeared after prolonged incubation with trypsin was probably due to growth on nutrients contained in trypsin preparations. Also, as seen in Table 17, lack of inhibition by thiomersalate of β-haemolysin which had interacted with red blood cells (in contrast to its inhibitory effect when added to titration diluents) agrees with the findings for α-haemolysin (Table 23). This evidence also supports the idea that certain structures, on large proteins and on the surface of erythrocytes compete with bacterial cell envelope components for haemolysin-binding.

However, the mechanism of synthesis and release of E. coli haemolysin remains a mystery. Two experimental approaches to this problem should aid in further characterisation of the proposed mechanism of release. First, valuable information could be gained by isolation and purification of the factors contained in culture media which are responsible for the appearance of α-haemolysin in culture filtrates. Second, addition of purified medium components to radioactively-labelled E. coli cells grown in chemically-defined medium should give information about both synthesis and release of haemolysin from these cultures. As demonstrated in this investigation, the ability to separate the haemolytic activities of E. coli by growth in selective media should prove advantageous for further studies of the relationship between α- and β-haemolysin.

In concluding this part of the discussion, it is useful to consider the findings of other workers in relation to the above results.

The findings of Snyder and Koch (1966) and Short and Kurtz (1971) which showed that α-haemolysin reached maximum levels between 6 and 10 hr when their strains were grown in complex medium suggest either, that the time course for release of α-haemolysin by different strains is different or, that different strains elaborate different haemolysins. Certainly, the findings of Smith (1963) regarding time of production and disappearance of haemolytic activity are compatible with the growth curve seen in Figure 6. Loss of haemolytic activity in culture (at 4 - 5 hr) may be related to heat-lability rather than inactivation by conditions of increased acidity or the presence of haemolysin inhibitors in culture media. That this loss did not occur when a small initial inoculum was used may reflect differences in the kinetics of growth and haemolysin release.

The suggestion of Muranyi and Juhasz (1971) that previous reports of filterable haemolysin were due to production of organic acids is not confirmed by observations made in this study. The findings of these workers, and the report by Snyder and Koch (1966) that filterable haemolysin produced in chemically-defined medium reached maximum levels when the culture was entering the stationary phase of growth and the pH was lowest (pH 5.2 - 5.4) does not agree with my results. In this study filterable haemolysin was not found in CDM even when cultures were assayed for activity for more than 20 hr. Moreover, the fact that β-haemolysin was routinely present in 0 hr cells grown in complex or chemically defined medium does not indicate a role for organic acids in haemolysis. Later sections will deal with the comparison of haemolysin purified in this study with that described by other authors.

C. Purification of α-Haemolysin.

As mentioned in the introduction to this thesis, the inability of previous workers to achieve considerable increases in specific haemolytic

activity (i.e. HU/mg) made purification of α-haemolysin a major aim of this thesis. Without highly purified haemolysin, results of biological tests may be suspect.

1. Methods used for purification of α-haemolysin.

The lability of crude α-haemolysin, even at 4°C, necessitated using procedures which could be done rapidly, reducing loss of haemolytic activity to a minimum. The inherent lability of α-haemolysin produced in complex medium might account for loss of haemolytic activity after dialysis of ammonium sulphate precipitates (Zwadyk and Snyder, 1971). These authors did not indicate for how long their preparations were dialysed.

In this study, complete precipitation of α-haemolysin was achieved at 50% (w/v) ammonium sulphate saturation. Prevention of inactivation due to prolonged storage at 4°C in a dialysis sac was the reason for relatively short dialysis (5 - 6 hr). Notably, precipitation of α-haemolysin produced in Bacto-Beef Heart for Infusion medium (Difco) was achieved by Zwadyk and Snyder at 30% (w/v) saturation with ammonium sulphate. The α-haemolysin produced in MEB and NBG in this laboratory did not precipitate until concentrations greater than 30% ammonium sulphate were added. At 50% saturation total recovered activity was always greater than the total amount of activity contained in culture filtrates. Although not discussed, Zwadyk and Snyder's results show also increased recovered activity after precipitation with ammonium sulphate. The fact that they first precipitated their haemolysin preparations in the cold with ethanol and used less ammonium sulphate might account for differences in recovered activity, i.e. 1.6 times the total activity present before precipitation with ammonium sulphate as compared to 3.5 times found in this study. Also, in this investigation up to 10-fold increases in total recovered activity were observed when haemolysin was precipitated by dialysing stage I haemolysin

against 0.005M acetate buffer at pH 4.6.

The apparent activation of α-haemolysin by "salting out" and precipitation at its iso-electric point will be discussed. It is sufficient for the moment to state that these methods allowed considerable purification of α-haemolysin as judged by increases in specific activity (see Table 20).

A summary of purification procedures used in this thesis is given in Table 28. Electrofocusing was of little value for purification of α-haemolysin, not due to low recoveries (procedure 4) but because of only small increases in specific activity, precipitation of α-haemolysin at its iso-electric point and the possibility that artifacts could emerge during draining of the column. To my knowledge only one other report has appeared describing purification by electrofocusing of cytolytic proteins from gram-negative bacteria. Wretlind, Mollby and Wadström (1971) have achieved separation by electrofocusing in a glycerol gradient, of a haemolysin isolated in culture supernatants of Aeromonas hydrophilia, into two components with pI's of 4.3 and 5.5. They also observed precipitation in acidic fractions after electrofocusing. However, pre-treatment of supernatant fluids by adsorption with DEAE Sephadex A-25 and polyethyleneglycol, and dialysis against 1% glycine allowed consistent and satisfactory separation of the 2 components. Electrofocusing studies of E. coli haemolysin, bearing these procedures in mind, may in future prove useful for rapid purification of this haemolytic protein.

Fractionation of stage II α-haemolysin on Sephadex G-200 in TSG buffer resulted in consistent recovery of highly purified haemolysin. The appearance of two peaks of activity only in the presence of glycerol is probably due to stabilisation of haemolysin molecules. The most likely explanation is that glycerol reduces the tendency to aggregate in solution.

TABLE 28. Summary of procedures used in the purification of *E. coli* α-haemolysin

Procedure	Haemolysin Preparation Used (stage)	Increase in Specific Activity	% Recovery*
1. 50% ammonium sulphate; precipitation and dialysis (stage I)	0	29.4	350
2. Dialysis against 0.005 M acetate buffer pH 4.6 (stage II)	I	900	375
3. Ultrafiltration with XM100A membrane	I	< 1	105
4. Electrofocusing in pH 3-10 sucrose column	I	3.2 (component with pI = 4.6)	70
5. Electrofocusing in pH 3-6 sucrose-urea column	I	2.3 (component with pI = 4.7)	2
6. Gel filtration on Sephadex G-200 in TS buffer	II	400 (peak fraction)	8
7. Gel filtration on Sephadex G-200 in TSG buffer (stage III)	II	4000 (peak fractions A and B pooled)	28

*In this table % recovery was calculated from the activity of the preparation at the preceding step in the purification procedure.

Although only 28% of the activity of stage II haemolysin was recovered after Sephadex chromatography, peak fractions contained up to 2.0×10^5 HU/ml and had a specific activity of $0.9 - 1.0 \times 10^6$ HU/mg. Such high activities have not been reported previously.

Assessment of the purity of stage III α-haemolysin proved difficult. Indeed, chemical analysis indicates that the haemolysin consists of at least 95% protein. The remaining 5% could not be accounted for by carbohydrate, nucleic acid or phospholipid; the presence of small amounts of lipids other than phospholipids cannot be excluded. Analysis of the amino acid composition would be the next step in characterising the chemical nature of the haemolysin.

Standard methods for assessment of purity such as SDS disc-gel electrophoresis were unsatisfactory due to the large molecular weight of α-haemolysin and its apparent reluctance to dissociate in the presence of SDS, urea or mercaptoethanol. Unfortunately, technical difficulties prevented analysis by ultracentrifugation.

The immunodiffusion microslide technique using antiserum prepared against stage II haemolysin was a useful analytical tool. The results shown in Plate I indicate the presence of two components in purified α-haemolysin. The minor precipitin line, which is adjacent to the antigen well, probably represents the medium component which was eluted at the void volume of Sephadex G-200 when sterile nutrient broth, processed to stage II, was applied to the column.

Zwadyk and Snyder reported inconsistent results using gel filtration on Sephadex and Biogel, adsorption to calcium phosphate and precipitation with zinc ions. As mentioned however, they were unable to

recover the haemolysin following dialysis of ammonium sulphate precipitates. In addition, although the appearance of two peaks of haemolytic activity observed by Short and Kurtz after fractionation of crude α-haemolysin on Sephadex G-200 (and on Sepharose 6B) correspond fairly well to the peaks of haemolytic activity observed in Figure 13, their resolution was poor in comparison. It is not possible to determine the extent of purification achieved by these authors as specific activities were not reported.

During purification and characterisation of α-haemolysin several important aspects of the structure and conformation of the molecule become evident and will now be discussed.

2. The structure of α-haemolysin.

Activation during purification: Assessment of the structure of a protein must naturally take into account external and internal forces applied upon it in solution. These include ionic interactions such as addition or removal of salts, inhibitors or activators, metal ion complexes and protein-protein interactions. In the case of E. coli α-haemolysin it must be remembered that, at present, the only method of monitoring activity is by lysis of erythrocytes. Since calcium ions are required for activity, it is therefore necessary to account for the structure of α-haemolysin in its unactivated and calcium ion-activated form.

The possibility cannot be excluded that activation during precipitation results from one of the following mechanisms:

(i) Loss of an inhibitor during the purification procedure. In this regard it would be interesting to combine fractionated supernatants with precipitated haemolysin to see whether there is a reduction in titre.

(ii) Alteration in molecular structure resulting in exposure of hidden active sites in the molecule or altered affinity for calcium ions.

Here it is worth pointing out that the binding of calcium ions by actomyosin, for instance, is markedly affected by changes in ionic strength (Weber and Herz, 1962; 1963); lower ionic strength favours the binding of calcium ions to myofibrils whereas high ionic strength lowers this affinity.

In stage I, precipitation by ammonium sulphate would be expected to cause increased protein-protein interactions and the resulting precipitate was therefore dissolved in distilled water.

(iii) The formation of biologically active subunits (see discussion of molecular weight).

At the moment it is not possible to decide whether 1 or more of these mechanisms is operating.

Molecular weight of α-haemolysin: The conclusions of Short and Kurtz (1971) about the molecular weight of α-haemolysin is not supported by their experimental results. They state that the molecular weight of the "smaller molecular weight species" from G-200 Sephadex was probably in excess of 3×10^5 based on its elution with the void volume from Sephadex G-100. It should be pointed out that the molecular weight exclusion limit of Sephadex G-100 is 1×10^5, not 3×10^5. Also, when substances are eluted with the void volume of Sephadex or other gel filtration methods conclusions can only be drawn regarding minimum molecular weights. This applies equally to my findings on Sephadex G-200.

The results presented in this thesis indicate that the usual methods for accurate estimation of sizes of proteins could not be applied to α-haemolysin. Fractionation on Sephadex G-200 gave only a minimum molecular size of 2×10^5. SDS disc-gel electrophoresis was unsuitable because failure of significant amounts of protein to enter 3.5% acrylamide gels made comparison with standard proteins impossible. It is worth noting here that Thorum and

Mehl (1968), who electrophoresed human γ-globulin (M.W. = 1.6 x 10^5) and fibrinogen (M.W. = 3.4 x 10^5) in 3.5% gels did not observe penetration of these proteins into the separating gel.

All of the methods used in this thesis indicate that the molecular weight of α-haemolysin is greater than 2.0 x 10^5. Diffusion coefficient analysis and direct measurement on electron micrographs of calcium ion-activated haemolysin (assuming an uncollapsed sphere) suggest a molecular weight of 5 - 6 x 10^5. The results of Amicon ultrafiltration of unactivated haemolysin indicate a molecular size of less than 3 x 10^5. However, it should be noted that pepsin (M.W. = 3.5 x 10^4) is reported by the Amicon Corporation as being retained by an XM50 membrane (exclusion at M.W. = 5.0 x 10^4). Also, the molecular weight value of 1.5 x 10^5 (assuming a collapsed sphere) obtained from electron micrographs of calcium ion-activated haemolysin suggests that the shape of the haemolysin molecule may be an important determinant in its diffusion through ultrafiltration membranes.

Even assuming a minimum molecular weight of 2 x 10^5, it appears that E. coli α-haemolysin is unique among extracellular cytolytic toxins of bacterial origin. Average molecular weights reported for most bacterial protein toxins are in the region of 2 - 6 x 10^4. Of course, the possibility cannot be discounted that the molecular weight of α-haemolysin represents, in part, a carrier protein which is firmly bound to the haemolysin molecule during liberation from E. coli cells (page 138). It would be desirable to purify such a carrier and determine its molecular weight. By subtraction it would then be possible to obtain an estimate of the true molecular size of α-haemolysin.

It is improbable that activation of the haemolysin during purification is due to the formation of biologically active subunits. Loss of haemolytic activity in the presence of urea indicates that the haemolysin is inactive in its unfolded state. Also, the evidence from disc-gel electrophoresis

suggests that even when dissociated in urea and SDS, the haemolysin has a large molecular weight, failing to penetrate 3.5% acrylamide gels. It is interesting to note that studies on β-galactosidase isolated from E. coli have shown that this enzyme consists of a biologically active tetramer (M.W. = 5.4×10^5; Craven, Anfinsen and Steers, 1965; Alpers et al., 1968) which is built up of 4 identical protomers (M.W. = 1.35×10^5) each consisting of a single polypeptide chain (Zipser, 1963; Ullman, Jacob and Monod, 1968).

D. The Kinetics of α-Haemolysin Activity.

The reaction of α-haemolysin with sheep erythrocytes was characterised in all experiments by an initial lag phase followed by a period of lysis which was linear between 20% and 80% haemolysis. By contrast, Zwadyk and Snyder (1971) concluded, by sampling at 1 min intervals, that the lag phase was effectively abolished at high concentrations of α-haemolysin. This discrepancy further serves to illustrate the importance of continuous monitoring of reactions, so that rate measurements can be determined accurately.

1. Activation by metal ions.

The study of activation by metal ions (M) of the biological activity of enzymes (E) suggests 3 possibilities.

1. $E + M + S = EMS$.
2. $E + M = EM$; $EM + S = EMS$.
3. $M + S = MS$; $E + MS = EMS$.

Mechanisms 2 and 3, in which the metal ion combines first either with the enzyme or the substrate (S), are most common. As pointed out by Dixon and Webb (1958), "determination of the mechanism is made much easier if there is a time effect in the activation of the enzyme". Such "time effects" for activation are common for peptidases and related enzymes which are activated

by manganese or cobalt ions, but are unusual for magnesium-activated enzymes.

Enough evidence now exists to conclude that calcium ions are required for both α- and β-haemolytic activity. However, previous investigations have provided little information on the function of calcium ions in the haemolytic reaction. Two findings show that the initial step in the haemolytic reaction consists of activation of the haemolysin by calcium ions.

a) Increased pre-incubation with calcium ions caused a reduction in the length of the lag phase (Figure 20).

b) Addition of EDTA after pre-incubation of haemolysin with calcium ions did not inhibit haemolysis (Figure 22).

The data given in Figure 18 indicate that a small amount of haemolysis occurred when red cells were incubated with haemolysin in the absence of exogenous calcium ions. That this is probably due to slow activation of haemolysin by erythrocyte-associated calcium ions is suggested by the finding that pre-incubation of haemolysin with red cells for longer than 5 min (Figure 21) was required to reduce the lag phase to a greater extent than was observed when haemolysin was pre-treated with calcium ions (Figure 20). To confirm this suggestion it would be necessary to determine the number of calcium ions required to activate a molecule of haemolysin, as normal erythrocytes contain only small amounts of calcium (10^{-17} moles/cell); (Weed and Lacelle, 1969).

The experiments using EDTA (Figure 22) indicate that calcium ions are <u>firmly</u> bound to the haemolysin molecule. Inhibition of haemolysis

occurred only when EDTA was added to haemolysin prior to activation by calcium ions. Also, the structural appearance of unactivated and activated haemolysin seen in negatively-stained electron micrographs is consistent with the view that the first step in the haemolytic reaction is the binding of calcium ions by haemolysin.

2. The haemolytic reaction.

The observation that the length of the lag phase was inversely proportional to the logarithm of the haemolysin concentration suggests that adsorption of activated haemolysin to red cells is required before haemolysis can occur. Furthermore, addition of EDTA during the lag phase to reaction mixtures containing calcium ion-activated haemolysin did not affect the haemolytic reaction. This apparently contradicts the observation of Short and Kurtz that addition of EDTA at any time during the haemolytic reaction prevented subsequent haemolysis. It should be emphasised that those workers used a different assay system. They did not pre-incubate haemolysin with calcium ions in the absence of erythrocytes; also they used considerably lower concentrations of haemolysin. It is still uncertain whether these differences can explain the inhibitory effect of EDTA. However it should be reiterated that, in my study, EDTA was inhibitory only when added to haemolysin without prior activation by calcium ions.

The finding that water-soluble phosphorus was not released from lecithin or sphingomyelin emulsions and that no products of hydrolysis could be detected using TLC indicates that α-haemolysin contains no phospholipase C activity. However, in view of reports describing the lysolecithinase activity of haemolytic El Tor vibrios (Felsenfield, 1944; Chatterje and Mitra, 1962), α-haemolysin should be screened for other enzymic activities.

The broad spectrum of haemolytic activity (Table 24) suggests that

α-haemolysin does not interact with specific phospholipid residues in red cell membranes. How then could binding of calcium-activated haemolysin take place? It is well known that the net surface charge of erythrocytes is negative. This is most likely made up of exposed negatively charged groups such as hydroxyl and keto groups on sialic acid residues, carboxyl groups on acidic amino acids and possibly hydrophilic phosphorylated groups of phospholipids (Hanahan, 1969). Since EDTA did not inhibit calcium-activated haemolysin, chelation of calcium ions by negatively charged groups on the surface of the red cell probably does not occur. A more likely possibility is that activation by calcium ions, which has been shown to cause changes in the structural appearance of the haemolysin molecule, exposes previously masked positively charged groups which bind to exposed negatively charged groups on erythrocyte membranes. It will be necessary to carry out a detailed study of the interaction of E. coli haemolysin with red cell ghosts and model lipid membrane systems to evaluate whether ionic or hydrophobic forces are dominant in the reaction.

The fact that the lag phase of haemolysis was inversely proportional to the logarithm of the haemolysin concentration is not consistent with a reversible enzymic mechanism of lysis. If an enzymic reaction was operating, one would expect an arithmetic relationship between lag phase and haemolysin concentration. Since the spectrophotometric assay necessitates using low red cell concentrations, the ratio of erythrocytes to haemolysin molecules was such that a "one hit" hypothesis could not be tested. However, if a molecular weight of approximately 4×10^5 daltons is assumed for the haemolysin, it can be calculated from Avogadro's number that 1 HU corresponds to 1.4×10^9 molecules and that about 150 molecules are required to lyse one erythrocyte in the tube titration test.

Studies of the mechanism of lysis by E. coli haemolysin are in their infancy. When one considers the fact that, with the exception of Cl. perfringens α-toxin and Staph. aureus β-toxin, the mechanisms of action of such well studied cytolytic toxins as the streptolysins, staphylococcal α- and δ-toxins and Cl. perfringens θ-toxin are not fully understood (Bernheimer, 1970), it must be expected that it will take some time to elucidate the action of E. coli haemolysin.

E. Biological Properties.

1. Studies in laboratory animals.

The results presented in this investigation raise the question of the role of E. coli haemolysin in pathogenicity. In contrast to the study of Smith (1963), who found that crude haemolytic culture filtrates, when injected intravenously, were lethal for 40 - 50% of mice and rabbits, partially-purified α-haemolysin produced by strain 25238 was non-toxic in these animals even when injected in considerably greater amounts (in terms of haemolytic units) than used by Smith. It is probable that his preparations contained large amounts of non-haemolysin medium components which might account for toxicity.

Similarities between α-haemolysin and heat-labile E. coli enterotoxin, with regard to their lack of toxicity after intravenous injection and their ability to cause intradermal swelling of rabbit skin, are most interesting. The time course for maximum swelling with α-haemolysin are in general agreement with observations made by Moon and Whip (1971) with LT preparations from porcine enteropathogenic strains of E. coli. It would be useful to inject rabbits with lower concentrations of Evans Blue following intradermal administration of α-haemolysin. I suspect that the concentration of dye used in these experiments was too great. Nevertheless, the fact that hard swellings developed in these animals and that the diameter of induration was proportional

to the amount of haemolysin injected suggests that common properties may exist between E. coli enterotoxin and haemolysin. At present, highly purified preparations of enterotoxin are not available. Only after the many factors contained in whole-cell lysates (i.e., LT) have been separated will it be possible to define the respective properties of these toxins.

A major criticism of the intestinal loop tests conducted in rabbits is that a positive reaction was not obtained with the Aberdeen β strain recorded as positive by Taylor et al., (1961). Nevertheless, the fact that high titre E. coli haemolysin has not previously been tested for enterotoxicity was sufficient reason for performing these studies.

In the light of recent investigations, it is not surprising that the enteropathogenic strain, serotype 055:B59(B5):H6, which was expected to give a positive dilatation reaction, was unreactive in intestinal loops. Other workers (Punyashthiti and Finkelstein, 1971; Gorbach and Khurana, 1972) have observed a similar non-reactivity with this serotype in rabbit ileal loops, even when fresh isolates were obtained from stools of patients with acute diarrhoea.

No fluid accumulation was seen in loops injected with α-haemolysin or strain 25238, indicating that haemolysin probably has no direct function in enterotoxaemia but, such loops appeared haemorrhagic (a feature not seen in controls injected with saline or heated toxin). This observation suggests that E. coli haemolysin may have a definite role in bowel 'oedema disease' of swine. Supporting evidence of other workers is as follows:

1. E. coli strains isolated from pigs affected with 'oedema disease' are invariably haemolytic.

2. E. coli strains isolated from these animals do not generally produce enterotoxins.

3. Heated supernatants of intestinal contents of affected pigs lose their capacity to evoke an experimental infection which mimics the natural disease.

4. Swine have high serum anti α-haemolysin titres.

As suggested by Smith and Halls (1968b), a direct, toxic effect is unlikely. It is proposed that destruction of intestinal epithelium by E. coli haemolysin allows invasion of the circulation by endotoxin components. This would account for the circulatory and neurological disturbances seen in natural and experimental infection. Substantiation of this hypothesis requires further extensive investigation. Histological studies, which were not performed because of the preliminary nature of these experiments, would obviously be desirable.

A role for E. coli haemolysin in 'oedema disease' such as suggested, would necessitate adsorption to epithelial mucosa. In this regard, it would be interesting to know if α-haemolysin is inactivated by ganglioside as has been shown to occur with cholera enterotoxin but not with E. coli enterotoxin. Further studies of E. coli haemolysin should prove fruitful in our understanding of intestinal and related diseases of domestic animals.

2. Role of haemolytic E. coli in infections of man.

The screening for haemolytic activity of E. coli strains isolated from inpatients and outpatients of 2 Glasgow hospitals suggests that the predominance of haemolytic strains isolated from infections of inpatients is due to the acquisition of these strains in the hospital.

It is obviously difficult to assess the significance of the results of a small survey. However, the frequent isolation of haemolytic E. coli

(40 - 70%) from infections of the buccal cavity, from urinary-tract infections and from wounds and abscesses, is worth noting. In view of the high percentage of haemolytic strains (70%) isolated from the area of the mouth and throat, it is interesting to speculate on how these strains are acquired. It is well known from the work of Smith and Halls (1967a; 1968b) that strains of E. coli isolated from domestic animals carry plasmids responsible for the production of enterotoxin (ENT) and haemolysin (Hly). A very recent paper has come to my attention in which evidence was found that E. coli of animal origin were reaching hospital patients via food (Hettiaratchy, Cooke and Shooter, 1973). Acquisition of Hly from animal strains by normally commensal E. coli may account, at least in part, for the high percentage of haemolytic strains isolated from this anatomical site.

Only 25% of strains isolated from faeces of patients with gastroenteritis were haemolytic, indicating perhaps that E. coli haemolysin is not a major virulence factor in human enteric infections. This does not exclude the possibility that the haemolysin, either acting alone or synergistically with other E. coli activities plays a role in some forms of human disease. Certainly the performance of a plate haemolysis test using the sheep erythrocyte overlay method described in this thesis might prove useful in the screening of potentially pathogenic E. coli.

The significance of finding high percentages of haemolytic strains which possess envelope antigens in common with particular enteropathogenic serotypes is not well understood. Dr. T. A. McAllister, who provided the strains of E. coli and the clinical data, has suggested that these findings might be related to possession by these strains of multiple drug resistance factors which could increase their ability to survive in the hospital.

Table 29 "Extracellular"toxins of gram-negative bacteria

Organism	Toxin	Isolation Procedure	Author
Bordetella pertussis	Heat-labile toxin	old cultures: freezing and thawing: sonic treatment:	Toomey and McClelland, 1933 Yamamoto et al., 1953 Verwey and Thiele, 1949
	Histamine-sensitising factor	supernatants from washed bacteria (3-4 days): sonic disruption:	Maitland and Guerault, 1958 Maitland, Kohn and Macdonald, 1955
Shigella dysenteriae	Exotoxin	alkaline extraction: autolysis:	van Heyningen and Gladstone, 1953 van Heyningen, 1971
Vibrio cholerae	Enterotoxin	filtrates of 6-12 cultures	De, Ghose and Sen, 1960 Burrows et al., 1965
V. cholerae (El Tor)	Heat-labile haemolysin	sonic disruption of 18 hr cells old cultures (cell autolysis)	Burrows et al., 1965 Watanabe and Felsenfield, 1961
V. parahaemolyticus	Heat-stable haemolysin	filtrates of young cultures	Sakazaki et al., 1968
Pasteurella pestis	Murine toxin	old cultures (cell autolysis)	Englesberg and Levy, 1954
Pseudomonas aeruginosa	Heat stable haemolysin	old cultures (cell autolysis)	Altenbern, 1966
	Fibrinolysin, protease, collagenase, lecithinase	filtrates of 18-24 cultures	Rangam et al., 1961; Heckly, 1970
Escherichia coli	Heat-labile enterotoxin	whole cell lysates	Gyles and Barnum, 1968
	Heat-stable enterotoxin	filtrates of 18-72 hr cultures	Smith and Halls, 1967c; Kohler, 1971b

F. "Extracellular" Products of Gram-Negative Bacteria : Perspectives.

Many cytolytic proteins have been isolated from gram-negative bacteria. Often, authors who have investigated these products have coined the terms "extracellular protein" or "exotoxin" to describe them. From Table 29, which gives a list of some toxins elaborated by gram-negative bacteria, it can be seen that in most cases these protein toxins are released from the cell only following mechanical disruption of cells or cell autolysis in old cultures. In fact, most of these products, with the possible exceptions of V. cholerae enterotoxin and the heat-stable haemolysin from V. parahaemolyticus, do not conform to the accepted criteria for extracellularity, i.e., that the maximum amounts of protein or enzyme should be liberated into the surrounding medium during logarithmic growth.

In my opinion, the findings of this thesis may provide a means to study other so-called extracellular products of gram-negative bacteria. In contrast to the isolation of true extracellular proteins and enzymes from most gram-positive bacteria, the liberation of extracellular products from gram-negative bacteria is probably prevented by virtue of the complex nature of the gram-negative cell envelope. Indeed, it would be interesting to see whether protein toxins and enzymes of other gram-negative bacteria could be removed from cells using suitable carrier or acceptor substances.

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SUMMARY

SUMMARY

Production of large amounts of high titre E. coli haemolysin has been achieved in a chemically-defined medium and in a glucose-nutrient broth medium. In chemically-defined medium only cell-associated, β-haemolysin was produced; in nutrient broth both extracellular α-haemolysin and β-haemolysin were found and, maximum yields of α-haemolysin were obtained within 2 hr after inoculation of cultures. More α-haemolysin was produced when large initial inocula were used but, loss of haemolytic activity occurred rapidly after maximum levels were reached.

Evidence is presented which suggests that α-haemolysin is a "released" form of β-haemolysin. Large molecular weight proteins, contained in nutrient broth, enhanced levels of α-haemolysin without affecting growth; both forms of haemolysin required calcium ions for activity, were inhibited by incubation with trypsin and were not affected by thiomersalate (in the case of β-haemolysin inhibition of haemolysis by thiomersalate was not observed after haemolytic E. coli cells had adsorbed to sheep erythrocytes).

Highly purified α-haemolysin was obtained by precipitation methods using 50% (w/v) ammonium sulphate (stage I), and dialysis against 0.005M acetate buffer, pH 4.6 (stage II), and by gel filtration on Sephadex G-200 at pH 7.3 in an eluant buffer containing 0.01M Tris, 0.1M NaCl and 5% (v/v) glycerol (stage III). No loss of haemolytic activity was observed following dialysis at any stage in the purification. Other purification procedures, such as electrofocusing, proved of little practical value. Using the above procedures, 76% of the total activity contained in crude culture filtrates was recovered and a 4000-fold increase in specific activity was achieved. This degree of purification has not previously been reported for E. coli haemolysin.

During precipitation procedures, activation of haemolytic activity occurred. Furthermore, in eluant buffer containing glycerol, the haemolysin was eluted from Sephadex G-200 near the void volume as two closely associated, but distinct peaks of activity. Several techniques were employed to estimate the molecular weight of α-haemolysin. Diffusion coefficient analysis ($D_{20} = 2.4 \times 10^{-7}$ cm^2 sec^{-1}) and direct measurements from electron micrographs of calcium ion-activated haemolysin indicated a molecular weight of $5 - 6 \times 10^5$ daltons. Other techniques such as SDS disc-gel electrophoresis and filtration through 'Diaflo' ultrafiltration membranes suggested a minimum molecular weight of 3×10^5 daltons. The activation of haemolysin during purification, its elution properties from Sephadex G-200 in the presence of glycerol and variations in molecular weight determinations are discussed in relation to the structural appearance of E. coli haemolysin in an unactivated and calcium ion-activated state.

Calcium ions are required for haemolytic activity. This was shown by continuous spectrophotometric monitoring of haemolysis which also indicated that the first step in the reaction is binding of calcium ions to the haemolysin (activation). An inverse relationship between the lag phase and the logarithm of the haemolysin concentration plus the fact that EDTA only inhibited haemolysis when added to haemolysin prior to activation by calcium ions, suggested that activated haemolysin was adsorbed to erythrocytes. The haemolysin had a broad spectrum of activity against various species of erythrocytes; sheep and rabbit red cells were the most sensitive. Phospholipase C activity was not detected in purified preparations of α-haemolysin.

Of 32 haemolytic E. coli strains tested by agar plate neutralisation using antiserum to stage II haemolysin, inhibition of haemolytic activity was observed in all cases. The haemolysin was non-toxic after intravenous

injection in mice and rabbits. High concentrations of haemolysin were required to produce a cytopathic effect in HeLa tissue culture cells. Hard swellings appeared when rabbits were injected intradermally with α-haemolysin; the diameter of the swelling was related to the amount of haemolysin injected. Dermonecrosis was not seen.

The α-haemolysin and its producing strain (25238) caused a haemorrhagic appearance in ligated intestinal loops prepared in rabbits. Saline and heated toxin (controls) produced no effect. In no experiment with any of the test agents was dilatation and fluid accumulation observed. These findings are discussed in relation to 'oedema disease' of swine.

A predominance of E. coli strains isolated from infections of inpatients from 2 Glasgow hospitals were haemolytic when compared to outpatients. Also, strains isolated from the buccal cavity, from urinary tract infections and from wounds were more often haemolytic than strains isolated from faeces. A possible role for E. coli haemolysin in pathogenicity is discussed.

APPENDICES

Appendix I

Media

A. Growth media:

1. Chemicaly defined medium (Snyder and Koch, 1966).

K_2HPO_4	2.3 g
KH_2PO_4	0.78 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$Na_3C_6H_5O_7 \cdot 2H_2O$	0.6 g
Distilled water	980 ml

Adjust pH to 7.3. Autoclave at 15 lb/in^2 for 15 min. Add 20 ml of a Millipore-filtered 10% (w/v) solution of α-D-glucose (i.e. 0.2% (w/v) final concentration).

Meat extract broth (Lovell and Rees, 1960; modified by Smith, 1963).

Fresh, macerated beef hearts	1 part by volume
Distilled water	2 parts by volume
Protease peptone (Difco)	1% (w/v)
NaCl	0.5% (w/v)

The pH of this mixture was adjusted to 7.8 with 1N NaOH. After autoclaving at 15 lb/in^2 for 10 min, the extract was filtered 2 to 3 times through layers of Whatman No. 1 filter paper (W. & R. Balston Ltd., England) until the filtrate was clear. After re-adjusting the pH to 7.6, the filtrate was autoclaved at 15 lb/in^2 for 10 min. Filter-sterilised glucose was added to this medium to a final concentration of 0.2% (w/v).

B. Assay media:

1. Sheep erythrocyte agar overlay medium:

Base: (per 100 ml).

Nutrient broth (Oxoid No. 2)	2.5 g
Bacto Agar (Difco)	1.5 g
Distilled water	98 ml

Autoclave at 15 lb/in^2 for 15 min. Cool to 50°C and add 2 ml of a filter-sterilised 10% (w/v) solution of α-D-glucose (0.2% (w/v), final concentration). Dispense 10 ml volumes into petri dishes and allow to harden. Remove surface moisture by incubating at 37°C for 30 - 60 min.

Erythrocyte overlay: (per 100 ml).

Solution 1:

Saline (0.85%, w/v)	80 ml
Bacto Agar (Difco)	1.5 g

Autoclave at 15 lb/in^2 for 15 min.

Solution 2:

Saline (0.85% w/v)	10 ml
$CaCl_2$	0.44 g
α-D-glucose	0.2 g

Sterilise by filtration through a 0.45 μ Millipore membrane.

Solution 3:

Sterile saline (0.85% w/v)	8 ml
Packed, washed sheep erythrocytes	2 ml

When solution 1 has cooled to 50°C, add solutions 2 and 3 and mix well. Pipette 10 ml aliquots of this mixture on top of the glucose-nutrient agar base. The final concentration of calcium chloride in the erythrocyte overlay is 0.04M.

2. Immunodiffusion assay medium:

Barbitone buffer pH 8.3 (see Appendix II)	50 ml
Distilled water	50 ml
Ionagar (Oxoid No. 2)	0.75 g

Steam to dissolve the Ionagar. Cool to 50°C and add 0.001% (w/v) thiomersalate. Dispense 10 ml volumes into universal bottles and store at 4°C until required.

Appendix II

Buffers and Diluents

1. Acetate buffer (after Cruikshank, 1969).

Stock solution A: 0.2M solution of acetic acid (11.55 ml glacial acetic acid in 1000 ml distilled water).

Stock solution B: 0.2M solution of sodium acetate (16.4 g $C_2H_3O_2.Na$ or 27.2 g $C_2H_3O_2Na\ 3H_2O$ in 1000 ml distilled water).

To prepare a 0.005M solution of acetate buffer, pH 4.6:

Solution A	25.5 ml
Solution B	24.5 ml
Distilled water to	2000 ml

2. Barbitone buffer pH 8.3.

Sodium barbitone (sodium diethyl barbiturate)	8.5 g
Distilled water to	450 ml
pH to 8.3 with 1N HCl (approximately 11.5 ml)	
Distilled water to final volume of	500 ml

3. Dulbecco's A saline.

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4 (anhyd)	1.15 g
KH_2PO_4 (anhyd)	0.2 g
Distilled water to	100 ml

This solution is 20 X concentrated and must be diluted 20-fold with distilled water before use.

4. Phosphate buffer.

Stock solution A: 0.2M solution of monobasic sodium phosphate (31.2 g $NaH_2PO_4.2H_2O$ in 1000 ml).

Stock solution B: 0.2M solution of dibasic sodium phosphate (71.7 gm $Na_2HPO_4.12H_2O$ in 1000 ml).

To prepare a 0.01M solution of phosphate buffer, pH 7.5

Solution A	16 ml
Solution B	84 ml
Distilled water to	2000 ml

5. Sodium cacodylate buffer, pH 5.0 - 7.4.

Stock solution A: 0.2M sodium cacodylate (42.8 g $Na\ (CH_3)_2AsO_2.3H_2O$ in 1000 ml).

Stock solution B: 0.2N HCl.

To prepare this buffer for use at a concentration of 0.01M

Solution A	25 ml
Solution B	1.4 ml (pH 7.4) to 23.5 ml (pH 5.0)
Distilled water to	500 ml

Calcium chloride at a final concentration of 10 mM (1.1 g $CaCl_2$ in 1000 ml) was added to this buffer for studies of the kinetics of erythrocyte haemolysis by α-haemolysin.

6. Tris (hydroxymethyl) aminomethane HCl (Tris HCl) buffer pH 7.2 - 9.0.

Stock solution A: 0.2M solution of Tris (24.2 g in 1000 ml)

Stock solution B: 0.2M HCl

To prepare this buffer for use at a concentration of 0.01M

Solution A	25 ml
Solution B	22.1 ml (pH 7.2)
	19.2 ml (pH 7.6)
	13.4 ml (pH 8.0)
	8.3 ml (pH 8.4)
	2.5 ml (pH 9.0)
Distilled water to	500 ml

Calcium chloride 10 mM (1.1 g $CaCl_2$ in 1000 ml) was added to this buffer for studies of the kinetics of erythrocyte haemolysis by α-haemolysin.

7. Veronal-calcium chloride (V-C) buffer, pH 7.3.

NaCl	8.5 g
Barbitone (diethyl barbituric acid)	0.575 g
Sodium barbitone	0.2 g
$MgCl_2.6H_2O$	0.17 g
$CaCl_2$	1.1 g (omit if not required)
Distilled water to	1000 ml

Appendix III

Electrofocusing solutions

Sucrose column (LKB 8101, 110 ml).

Dense electrode solution (anode):

	Sucrose 'Analar'	12 g
	Distilled water	14 ml
	Sulphuric acid (99% v/v - Hopkins and Williams)	0.2 ml

The sulphuric acid is added after dissolving the sucrose; the solution must be stirred to prevent carmelisation of the sucrose.

Dense solution for gradient:

	Sucrose 'Analar'	25 g
	Distilled water	32 ml
pH 3-10	Ampholine, 10% (w/v) in distilled water	7.5 ml
	Total volume	55 ml

Light solution for gradient:

	Distilled water	37.5 ml
	Haemolysin	15 ml
pH 3-10	Ampholine, 10% (w/v) in distilled water	2.5 ml
	Total volume	55 ml

Light electrode solution (cathode):

	Distilled water	20 ml
	Sodium hydroxide	0.2 g

Sucrose + 3.5M Urea column (LKB 8101, 110 ml).

Dense electrode solution (anode):

	Sucrose 'Analar'	12 g
	Urea 'Analar'	4.62 g
	Distilled water to 22 ml after dissolving sucrose and urea.	
	Add 0.2 ml sulphuric acid (99% v/v) with stirring.	

Dense solution for gradient:

	Sucrose 'Analar'	25 g
	Urea 'Analar'	11.55 g
	Distilled water to	47.5 ml
pH 3 - 6	Ampholine 10% (w/v) in 3.5M urea	7.5 ml
	Total volume	55 ml

Light solution for gradient:

	Urea 'Analar'	11.55 g
	Distilled water to	42.5 ml
	Haemolysin	10 ml
pH 3 - 6	Ampholine 10% (w/v) in 3.5M urea	2.5 ml
	Total volume	55 ml

Light electrode solution (cathode):

Urea 'Analar'	4.2 g
Sodium hydroxide	0.2 g
Distilled water to	20 ml

All solutions were prepared just prior to electrofocusing and were kept at $4^{o}C$ until preparation of gradients in the column.

Appendix IV

SDS Disc-Gel Electrophoresis

Stock solutions.

A. Separation Gel Solution:

Acrylamide	1.75 g
BIS (NN'-methylene-bisacrylamide)	0.10 g
Glacial acetic acid 'Analar'	1 ml

pH to 4.9 with 1M potassium hydroxide

Urea 'Analar'	27.02 g
SDS 20% (w/v) in distilled water	5 ml
Distilled water to	50 ml.

B. Riboflavin Solution:

Riboflavin	0.04 g
Distilled water to	10 ml

C. Persulphate Solution:

Ammonium persulphate	0.5 g
Distilled water to	10 ml

D. TEMED (NNN'N'-tetramethylene diamine).

E. 20% sodium dodecyl sulphate (SDS) (Koch Light) in distilled water.

F. Destaining Gel Buffer:

1N HCl	48 ml
Tris	5.98 g
TEMED	0.46 ml
Distilled water to	100 ml
pH 6.7	

G. Destaining Gel:

Acrylamide	16 g
BIS	4 g
Distilled water to	100 ml

H. 40% sucrose in distilled water.

Preparation of Separating Gel:

Solution A	12.5 ml
Solution B	0.125 ml
Solution C	0.125 ml
Solution D	0.01 ml
Solution E	0.03 ml

Mix well, pipette 1.5 ml volumes into disc-gel tubes sealed at one end with parafilm and carefully layer each column with water. Photopolymerise the gels for 30 - 45 min.

Electrophoresis Tank Buffers.

Upper tank buffer:

Glycine 'Analar'	8.3 g
Glacial acetic acid 'Analar'	0.6 ml
pH to 4.9 with 1M potassium hydroxide	
Urea 'Analar'	360.4 g
Distilled water to	1000 ml

Lower tank buffer:

KOH	3.37 g
Glacial acetic acid 'Analar'	265 ml
pH to 2.7	
Distilled water to	1000 ml

Add 22 ml of 20% (w/v) SDS to 220 ml of upper and lower tank buffers before electrophoresis.

Fixative and Stain:

Amido Black (Gurr, London)	1 g
Glacial acetic acid 'Analar'	10 ml
Methanol 'Analar'	50 ml
Distilled water	40 ml

This solution was filtered before use.

Preparation of Destaining Gel:

Solution F	1.5 ml
Solution G	3.0 ml
Solution B	1.5 ml
Solution H	6.0 ml

Mix well, layer 0.5 ml volumes into parafilm-covered destaining tubes and photopolymerise for 30 min.

Electrode polarity:

In all electrophoresis runs, the cathode was connected to the upper terminal and the anode to the central terminal.

Standard proteins:

Bovine serum albumin: Sigma, St. Louis, U.S.A.
molecular weight = 67,000.

Yeast hexokinase: Sigma, St. Louis, U.S.A.
molecular weight = 45,000
Tetramer molecular weight = 96,000.

Ovalbumin: Koch-Light, Colnbrook, England
molecular weight = 45,000.

Bovine pancreatic chymotrypsinogen: Miles-Seravac, Maidenhead, England
molecular weight = 25,000.

Horse heart cytochrome C: Koch-Light, Colnbrook, England
molecular weight = 12,400.

Appendix V

Reagents for Chemical Analysis

A. Estimation of carbohydrate.

1. Nelson method (Nelson, 1944).

Reagent A.

Na_2CO_3 (anhyd)	12.5 g
KNa tartrate	12.5 g
$NaHCO_3$	10.0 g
Na_2SO_4 (anhyd)	100 g
Distilled water to	500 ml

Reagent B.

$CuSO_4 \cdot 5H_2O$	7.5 g
Distilled water to	50 ml
Sulphuric acid (99% v/v - Hopkins and Williams)	1 drop

Arsenomolybdate Reagent.

a) $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 25 g

Distilled water to 450 ml

Add 21 ml of sulphuric acid, 99% (v/v).

b) $Na_2HASO_4 \cdot 7H_2O$ 3.0 g

Distilled water to 25 ml

Add b) to a) and store in brown bottle for 24 hr at 37°C.

Reagent should be yellow with no green tint.

2. Anthrone method (Scott and Melvin, 1961).

Reagent

Anthrone	2 g
Sulphuric acid (99% v/v - Hopkins and Williams)	1000 ml

B. Estimation of Phosphorus (Allen, 1940).

Standard phosphate solution

KH_2PO_4 (anhyd)	2.193 g
Distilled water to	500 ml

1.0 ml contains 1 mg PO_4

Reagents

10NH_2SO_4 :- 280 ml concentrated H_2SO_4 (MAR grade) to a final volume of 1 litre.

1% Amidol in 20% sodium metabisulphite :-

1 g of amidol dissolved in 100 ml of 20% (w/v) sodium metabisulphite and filtered. Store in dark brown bottle and prepare daily as required.

8.3% (w/v) ammonium molybdate

H_2O_2 100 vol MAR grade.

C. Estimation of DNA (deoxyribonucleic acid) (Burton, 1956).

Diphenylamine reagent:

diphenylamine	1.5 g
Glacial acetic acid 'Analar'	100 ml
Sulphuric acid (99% v/v - Hopkins and Williams)	1.5 ml

Store this solution in the dark. Before use add 0.1 ml of aqueous acetaldehyde (16 mg/ml) per 20 ml of the diphenylamine reagent.

What hath night to do with sleep?

John Milton, Comus l. 122.